STUDIES OF VITAMIN B_{12}, CHOLINE AND RELATED FACTORS IN THE DIETS OF CHICKS [1]

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Investigations some years ago showed that choline, when added to a purified basal diet (Jukes, '40), would prevent perosis in chicks and increase their rate of growth. Methionine and betaine were ineffective although certain analogs of choline, such as arsenocholine, could substitute adequately. These and other findings reported in the literature were reviewed by Jukes ('47).

The relation of vitamin B_{12} and folic acid to the response of the chick to choline and other "methylating" compounds has become of interest. Schaefer and co-workers ('49a, b) studied the growth of chicks on a diet which contained added folic acid but was deficient in both vitamin B_{12} and methionine, and found that growth was more rapid when the level of choline was 6 gm per kilogram of diet than when the level was 1 gm. However, when a preparation containing vitamin B_{12} was added, growth was as rapid with 1 gm of choline per kilogram of diet as with higher levels. It was subsequently noted (Schaefer et al., '50) that when vitamin B_{12} was included in the basal diet the level of choline required for growth was greater in the absence of added folic acid than in its presence.

Gillis and Norris ('49a) compared the effects of various supplements on the growth rate of chicks which received a basal diet consisting principally of cereals and soybean meal.

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A fair growth response was obtained with 2 gm of either betaine or choline per kilogram of diet, but a better response was produced by 1.5 gm of a "liver paste" containing about 17 μg of vitamin-B_{12} activity per gram. No additional growth response to betaine or choline was obtained when the liver paste was included in the diet. Similar results were obtained when vitamin B_{12} was used instead of liver paste (Gillis and Norris, '49b).

The possible function of glycine and serine as precursors of "labile methyl" groups via formate has aroused interest as a result of studies with rats by Sakami ('49), Siekevitz and Greenberg ('50), Welch and Sakami ('50), du Vigneaud et al. ('50) and others. Metabolic reactions involving formate, glycine and serine were slowed by a deficiency of folic acid (Elwyn and Sprinson, '50; Plaut et al., '50; Totter et al., '50).

It was reported that a deficiency of choline in rats may be alleviated by vitamin B_{12} and folic acid in the diet (Bennett, '49), presumably by facilitating the formation of choline from glycine and serine (Stekol and Weiss, '50).

Choline deficiency in chicks was found to be aggravated by adding a dietary source of glycine either as the free amino acid (Jukes, '41a) or as gelatin (Jukes, '41b). Although special precautions were not taken in these earlier experiments to supply vitamin B_{12} and folic acid, the chicks were hatched from eggs laid by hens fed mash containing 12.5% of sardine fish meal and the basal choline-deficient diet used for the chicks contained 5% of yeast. It was found by McGinnis and Carver ('47) that the inclusion of 4.6% of fish meal in the maternal diet was sufficient to protect chicks from a deficiency of a growth factor, later shown to be vitamin B_{12}, during the first 4 weeks following hatching. Yeast is known to be a good source of folic acid. It would, therefore, appear unlikely that marked deficiencies of vitamin B_{12} and folic acid were encountered in the earlier investigations by Jukes ('40, '41a). In the present investigation the effects of choline and other

compounds on growth and perosis in chicks were compared in the presence and absence of vitamin B_{12}.

EXPERIMENTAL

Chicks (Barred Rock × New Hampshire) were obtained from eggs laid by hens on an all-vegetable diet described previously (Stokstad et al., '49) and were fed the experimental diets immediately. They were examined for perosis at intervals and signs of this bone deformity were usually found to appear at the age of 7 to 10 days in chicks on the basal choline-deficient diet. The incidence reached approximately a maximum at the age of 19 days, so that this date was arbitrarily selected for scoring groups for comparative purposes.

Choline and betaine were fed as the chloride and hydrochloride, respectively, and the weights of these supplements are expressed in terms of these salts.

Vitamin B_{12} was administered by intramuscular injection of a solution containing a mixture of vitamins B_{12} and B_{12b} unless otherwise indicated. The solution was found to supply sufficient vitamin B_{12} to restore normal growth in deficient chicks on a corn-soybean meal diet when injected at a rate of 0.3 μg of total vitamin B_{12} weekly. From 10 to 15 chicks were used in each group.

The composition of diet 1, deficient in choline, is shown in table 1. The results of three experiments carried out with this diet are shown in table 2. The results of experiment 1 indicated that when the chicks were deficient in vitamin B_{12} fairly good growth was obtained by adding choline at a level of 3 gm per kilogram of diet; 0.6 gm was insufficient and 6 gm depressed growth. With vitamin B_{12} supplied, 0.6 gm of choline was almost sufficient for growth but would not prevent perosis. The addition of betaine to the diet containing a suboptimum level of added choline did not improve growth or prevent perosis in the presence of vitamin B_{12}. In experiment 2, betaine appeared to improve growth slightly in the absence of vitamin B_{12} but methionine was ineffective. Bet-

aine had only a slight effect in experiment 3 when it was added to a diet containing 0.3 gm of choline per kilogram of diet. No antiperotic effect of betaine was noted in any of the experiments when vitamin B_{12} was supplied. The incidence of perosis was greater in the presence than in the absence of vitamin B_{12} when insufficient levels of choline were

TABLE 1

Composition of diet 1 (purified diet, deficient in choline) and diet 2 (deficient in methionine and vitamin B_{12})

	DIET 1	DIET 2		DIETS 1 AND 2
	gm	*gm*		*mg*
Glucose (cerelose)	58.5	15.4	Niacinamide	5
Ground green split peas		70		
Washed casein	21		Calcium pantothenate	5
Gelatin	8	8	Thiamine HCl	1
Calcium gluconate	5		Riboflavin	1
Corn oil + vitamins A, D and E [1]	3	1	Pyridoxine HCl	1
Bone ash	2	3	1-acetoxy-2-methyl-4-naphthyl sodium phosphate	0.5
Salt mixture [1]	2	1	Pteroylglutamic acid	0.2
Sodium chloride		1	Biotin	0.02
Cystine	0.4			
Choline chloride		0.2		
Inositol	0.1	0.1		
DL-tryptophan		0.2		

[1] See Stokstad et al. ('49).

fed. Perosis was noted at all levels of choline below 1.5 gm which were tested, and although 0.7 gm of added choline appeared to be almost sufficient to supply the requirement for growth in the presence of vitamin B_{12}, this level was not sufficient to prevent perosis.

Various levels of vitamin B_{12} were added to the diet in the presence of two different levels of choline in experiment

4 and the results are shown in table 3. The growth-promoting effect of vitamin B_{12} was more marked at the low level of choline than at the high level, but once again it was noted that perosis occurred at the lower level of choline.

TABLE 2

Effects of vitamin B_{12} on the response obtained to choline and other supplements from chicks on a purified choline-deficient diet (Diet 1)

EXPERIMENT	SUPPLEMENT PER KG OF DIET	WEIGHT AT 28 DAYS		% PEROSIS AT 19 DAYS	
		Without B_{12}	With B_{12}[1]	Without B_{12}	With B_{12}[1]
		gm	gm		
1	None	104	152	67	92
2	None	89	163	80	100
3	None	114	153	50	90
3	0.3 gm choline	136	208	50	60
3	0.3 gm choline + 1 gm betaine	155	223	30	50
1	0.6 gm choline	171	295	50	50
1	0.6 gm choline + 2 gm betaine	190	285	8	50
2	0.7 gm choline	159	302	7	7
3	0.7 gm choline	160	332	0	20
2	0.7 gm choline + 2 gm betaine	183	286	0	20
2	0.7 gm choline + 2 gm methionine	144	321	0	40
3	1.5 gm choline	225	317	0	0
2	3.0 gm choline	160	337	0	0
1	3.0 gm choline	232	324	0	0
1	6.0 gm choline	128	245	0	0
	Average incidence of perosis in all groups without B_{12}			23%	
	Average incidence of perosis in all groups with B_{12}			39%	

[1] One microgram injected weekly as a mixture of vitamins B_{12} and B_{12b}.

TABLE 3

Effect of various levels of vitamin B_{12} and choline on chicks receiving diet 1

B_{12} ADDITION PER KG OF DIET 1	WEIGHT AT 28 DAYS		PER CENT INCIDENCE OF PEROSIS AT 23 DAYS	
	0.6 gm choline	3 gm choline	0.6 gm choline	3 gm choline
	gm	gm		
None	131	226	10	0
5 μg	216	240	60	0
10 μg	211	275	30	0
20 μg	259	277	63	0

The composition of diet 2, a diet of natural foods which was devised to be deficient in methionine but reasonably adequate in other respects, is shown in table 1. Vitamin B_{12}-deficient chicks were fed the diet with the supplements, and the results are shown in table 4. No evidence for a sparing effect of vitamin B_{12} on the methionine requirement was noted and the growth response to vitamin B_{12} was greater at higher levels of methionine than at lower levels.

An attempt was made to repeat the experimental conditions of Gillis and Norris ('49a). Chicks were obtained from

TABLE 4

Effects of methionine and vitamin B_{12} on the growth of chicks which received diet 2

EXPERIMENT	AMOUNT OF DL-METHIONINE ADDED PER KG OF DIET 2	WEIGHT AT 28 DAYS		INCREASE WITH B_{12}
		Without B_{12}	With B_{12}[1]	
		gm	*gm*	*gm*
5	None	72	102	30
6	None	82	109	27
5	2 gm	137	236	99
5	4 gm	178	311	133
6	4 gm	144	286	142
6	5 gm	175	299	124
6	6 gm	165	314	149

[1] One microgram injected weekly as a mixture of vitamins B_{12} and B_{12b}.

a commercial hatchery and were placed on basal diet 3, which had the composition described by Gillis and Norris. At three weeks of age the chicks were divided into groups and supplements were added to diet 3. The results are shown in table 5, and indicate that little or no deficiency of choline was encountered. This might have been anticipated from the choline content of the diet, which was calculated to be about 1.3 gm per kilogram on the basis of values cited by Jukes and Schneller ('49), but variations in the choline content of natural foods might well cause a variation from this calculated value.

TABLE 5

Growth of chicks on basal diet[1] of natural foodstuffs (Gillis and Norris, '49a) with various supplements

SUPPLEMENT PER KG OF DIET	GAIN IN WEIGHT 21 DAYS TO 42 DAYS	
	Without B_{12}	With B_{12}[2]
	gm	*gm*
None	255	316
2 gm betaine HCl	264	326
2 gm choline chloride	291	326
3 gm DL-methionine	276	341
Average weight per chick in all groups at 21 days:	174 gm	

[1] Corn, wheat, oats, soybean meal (30%), A and D oil, and minerals, plus riboflavin 0.1 gm and calcium pantothenate 0.25 gm per 100 lb.

[2] One-half microgram injected weekly.

DISCUSSION

The results showed that chicks had a comparatively high requirement for dietary choline even when they were well supplied with methionine, glycine, serine, pteroylglutamic acid and vitamin B_{12}. The presence of an inadequate level of choline resulted in the appearance of perosis and the onset of this condition was slower when vitamin B_{12} was not supplied. Betaine had very little effect when fed with suboptimum levels of choline.

No sparing effect of vitamin B_{12} upon the methionine requirement of chicks was noted when a diet was fed which was supplemented with choline, pteroylglutamic acid and other vitamins.

The statement was made by Stekol and Weiss ('50) that it had been reported by Gillis and Norris ('49a) "that the 'animal protein factor' concentrate makes dietary choline unnecessary in animal organism" *(sic)*. Neither the report by Gillis and Norris nor the present investigation permits such a conclusion.

SUMMARY

1. Choline deficiency as produced by a purified diet was studied in chicks which were also deficient in vitamin B_{12}.

The requirement of choline for maximum growth was found to be greater in the absence of vitamin B_{12} than in its presence. However, the amount of choline required for the prevention of perosis was not decreased by supplying vitamin B_{12}.

2. Betaine did not have a marked effect when added to diets containing an insufficient level of choline.

3. A methionine-deficient diet was fed to vitamin B_{12}-deficient chicks and no evidence of a sparing effect of vitamin B_{12} on the methionine requirement was noted under the experimental conditions encountered.

4. Choline deficiency was not encountered on a diet of natural foods containing a high level of soybean meal.

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EXCRETION OF CA^{45} INTO THE GASTROINTESTINAL TRACT OF YOUNG AND MATURE RATS[1]

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TWO FIGURES

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For many years there has been much controversy in the literature concerning the excretory role of the gastrointestinal tract as it relates to calcium metabolism. Some workers have found no evidence of an active excretion, while others have accepted the view that the large intestine in particular has an important function in regulating the blood level of certain minerals. Findings with the various species have not been in good agreement, indicating a possible species difference in this regard.

Johnson ('37) reported that the colon of patients with colostomy did not excrete significant quantities of calcium. McCance and Widdowson ('39) found no evidence that additional calcium was excreted into the gastrointestinal tract of normal humans following intravenous injection. In mineral studies with rats, Henry and Kon ('39) revealed no active excretion of calcium into the large intestine. Adolph and Liang ('41) suggested that apparently in the rat the intestinal tract does not excrete calcium in amounts which are related to the metabolic calcium level. The effects of intra-

[1] Published by permission of the Director of the University of Florida Agricultural Experiment Station.

venous injections into rats on both high and low calcium diets led them to this belief.

Bergeim ('26) stated that there was a marked excretion of calcium into the lower bowel of rachitic rats, while in normal animals there appeared to be an approximate balance between excretion and absorption. However, this work revealed no proof that excretion actually took place. Interesting work by Cowell ('37) in which individual fecal pellets of rabbits were analyzed provides good evidence that in this species the colon excretes important quantities of calcium. Pecher ('41) injected radioactive calcium into mice and found that substantial quantities appeared in the digestive tract. Further evidence for the excretion of calcium is provided by Greenberg ('45). Steggerda and Mitchell ('46) have shown that subjects on low calcium diets may excrete twice as much calcium as is consumed, following the administration of citrate. This is good evidence in favor of an active calcium excretion. Numerous other investigators have provided indirect evidence that excess blood calcium, as well as other mineral elements, are excreted by the gastrointestinal tract.

The work reported here was undertaken to help clarify this seemingly unsettled question, and to study the influence of age on the retention of calcium.

EXPERIMENTAL

Fourteen hooded rats of the Long-Evans strain served as experimental animals. They consisted of two groups of 7 each. Rats in one group were 40 days old and averaged 70 to 80 gm in weight. Mature 370-day-old rats, weighing from 250 to 280 gm, comprised the other group. The latter were females, all of which had raised one litter of young. All rats in both groups were considered normal and in a good state of calcium nutrition. During the experimental period, they were fed ad libitum a complete diet [2] which they had received for a period of two weeks before the experiment commenced.

[2] Staf-O-Life dog pellets.

Approximately 17 microcuries of radioactive calcium[45] as $CaCl_2$ in 1 ml of aqueous solution were administered intramuscularly in the thigh of each animal. After definite intervals of time (figs. 1 and 2), rats from each group were sacrificed by ether anesthesia. The entire gastrointestinal tract was removed and carefully cleaned of adhering connective tissue. The tract was then divided into the desired segments with the upper one-fourth of the small intestine representing the duodenum. The contents of each segment were carefully removed by washing with distilled water. The mucosa was separated from the muscularis of each segment by the use of a blunt-edged knife and a gentle scraping technique. Quantitative urine and fecal collections were initiated at the time of injection and continued until the animals were sacrificed.

All samples were wet digested with concentrated nitric acid to low volume, filtered, and made up to volume. The oxalate procedure for radioactive assays (Shirley et al., '50) was followed in all determinations. Thin mica-window (1.5 to 2.0 mg per square centimeter) Geiger-Mueller tubes were used in conjunction with commercial scalers for the activity measurements. The surface area of all precipitates was 2.835 cm^2.

RESULTS AND DISCUSSION

Findings relative to the concentration of Ca^{45} in the tissue of the various segments are presented in figure 1. The data reveal that significant quantities of the isotope appeared in the tissue of all segments as early as 15 minutes after administration. Approximately one-third of the amount of the isotope found in the alimentary tissues was present in the mucosa, the remaining portion in the muscularis. There were no significant variations between the different segments in this regard. The peak of the concentration occurred sooner, and appreciable quantities of isotope were present during a shorter period of time, in the case of the young rats. Possibly this was a consequence of a more intensive metabolism of the young animals or indicative of a greater wastage of

calcium on the part of the mature animals. After a period of two weeks small quantities of the isotope still appeared in the mucosa and muscularis of the intestinal tract of both age groups. The relative concentration in the various segments was approximately the same at all intervals after administration. These data do not reveal a predominating function of

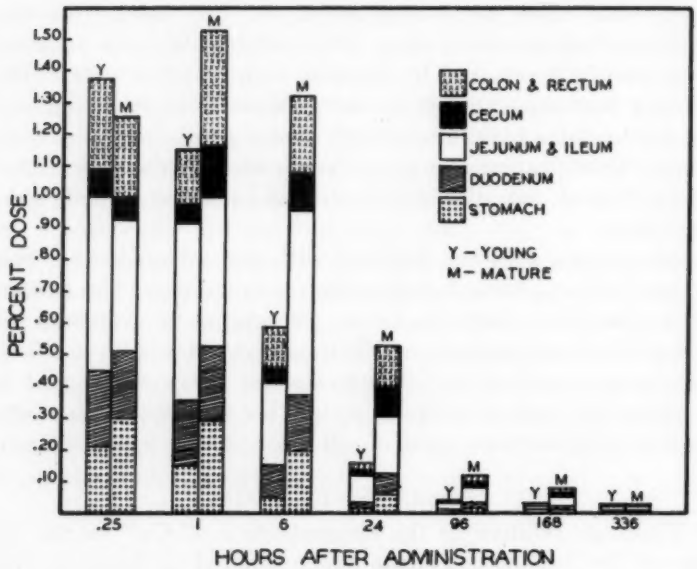

Fig. 1 Ca^{45} in tissue of digestive tract.

the large intestine in the excretion of calcium. Rather they suggest an excretion of the mineral in all parts of the tract.

The distribution of Ca^{45} in the contents of the various segments of the alimentary tract is presented graphically in figure 2. At the 15-minute and one-hour intervals, the contents of the tracts of the two age groups showed a concentration similar in relative distribution to that found in the tissue of the corresponding segments at the same time intervals. During these short time intervals a large part of the activity

was found in the contents of the small intestine of both groups, thus indicating that the large intestine plays no predominating role in the excretion of calcium by the rat. Six hours and later after administration of the isotope the greatest concentration occurred in the large intestine, which would be expected in view of the passage of the contents along the tract. Subsequent to the 6-hour period a striking difference existed between the young and mature rats, in that the young rats had appreciably less isotope present in the contents of the

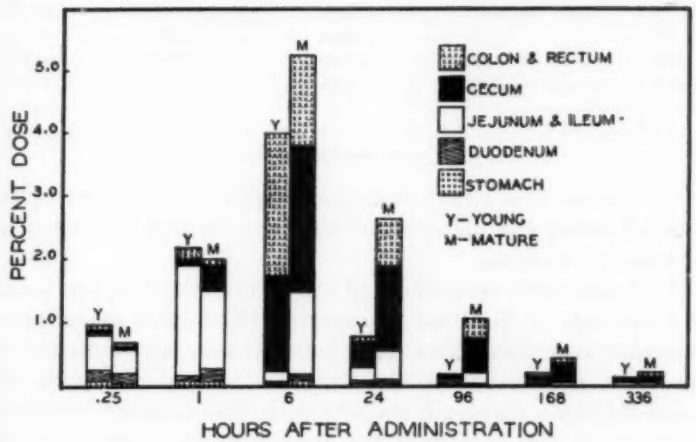

Fig. 2 Ca^{45} in contents of digestive tract.

gastrointestinal tract, indicating that they must reabsorb the element in greater quantities than the more mature animals. The fecal data shown in table 1 confirm this difference, since the mature rats excreted 41.6% of the dose in the feces during the two-week period, compared to 15.2% for the young rats. The young rats might be expected to retain more calcium for growth purposes and would therefore excrete less in the feces. These data suggest that the cecum and large intestine are mainly concerned in the reabsorption of calcium. As is shown in table 1, essentially no difference occurred in

the excretion of the calcium isotope in the urine of the two age groups.

TABLE 1

Urinary and fecal recovery of Ca^{45}

HOURS AFTER ADMINISTRATION	YOUNG RATS		MATURE RATS	
	Urine % dose	Feces % dose	Urine % dose	Feces % dose
½	0.00	0.00	0.00	0.00
1	0.00	0.04	0.00	0.84
6	1.08	0.49	0.38	0.20
24	0.56	6.60	2.21	10.26
96	..	9.31	1.35	28.54
168	3.07	12.49	3.59	35.27
336	1.66	15.22	2.10	41.55

SUMMARY

1. The excretory rate of various segments of the intestinal tract of young and mature rats has been studied by the use of radioactive calcium.

2. Appreciable quantities of Ca^{45} were found in the tissue and contents of the tract as early as 15 minutes after intramuscular injection. The data indicate that all segments of the tract participate in the excretion of calcium with the small intestine playing a major role in this capacity, which is contrary to the view that the large intestine predominates in this function.

3. Fecal recovery indicated that extensive excretion of the isotope had occurred by way of the alimentary tract. After a two-week period, 15.22 and 41.55% of the dose were recovered in the feces of the young and mature rats, respectively.

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THE ABSORPTION OF FATTY ESTERS IN THE MOUSE INTESTINE [1]

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The papers of Frazer appearing from 1939 to the present (Frazer, '48) have indicated that the triglycerides of the long chain fatty acids are only partially hydrolyzed in the slightly acidic environment of the small intestine and are for the most part absorbed as emulsions into the lacteals, arriving in the systemic blood through the thoracic duct. The nature of this emulsion was found to be very important. In order for adequate absorption to take place it had to be composed of negatively charged particles of less than 0.5 μ in diameter. *In vitro* experiments indicated that at the pH of the duodenum (pH 6.5), the only combination of substances normally occurring in the small intestine capable of forming such an emulsion was that of fatty acids, bile salts and monoglycerides. *In vivo* work, however, has proved the necessity of the presence of bile salts only. The presence in the intestine of partially hydrolyzed glycerides was shown, but the necessity of their presence was not proved (Frazer, '48).

Present work in this laboratory has involved the use of a methyl ester of a fatty acid for absorption studies. The question therefore arose as to the method of absorption of such compounds which could not give rise to partially hydrolyzed glycerides. A program was consequently undertaken not only

[1] This paper is based on work performed under Contract AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

to determine the mechanism of absortion of the fatty esters, but also to elucidate further the requirements for emulsion formation during triglyceride absorption. Some previous work has been carried out on these substances. Frank (1898) found that the ethyl esters of the long chain fatty acids were totally hydrolyzed before absorption, while Miller, Barnes, Kass and Burr ('39) found the absorption of the methyl esters of the conjugated acids derived from corn oil to be abnormal. It should be pointed out that both these groups used fasted animals in their experiments. The present study not only indicates that this is an abnormal state, but gives some further insight into the mechanism of fat absorption in general.

EXPERIMENTAL

Materials

The mice used in the experiments were mature albino females of the CF_1 strain, raised in the project animal colony. The methyl 9, 11-octadecadienoate was prepared in these Laboratories [2] (see appendix).

Methods

For studies on emulsion formation in the small intestine, mice were given about 70 mg of oil, using a tuberculin syringe with a bent No. 19 needle ending in a blunt tip of silver solder. After one hour the animals were sacrificed and the small intestines removed and washed out with several portions of distilled water. The suspensions were filtered through a loose plug of glass wool and centrifuged at about 100 *g* for 10 minutes. They were then made up to 40 ml for turbidity measurements.

Turbidities of the intestinal washings were measured at 90° to the incident light, using the Phoenix B-S Light Scattering Photometer. In each measurement the instrument was standardized against a reference standard of known transmittance.

[2] The methyl oleate was obtained from the Hormel Foundation and the monopalmitin from the Armour Laboratories.

The readings so obtained could therefore be compared directly to one another; and thus a comparison of the degree of emulsification could be made.

For measurement of the total amount of absorption, 10% of the spectroscopically active ester, methyl octadecadienoate, was included in the methyl oleate; and the amounts remaining in the stomach and intestines were determined quantitatively by washing out the contents of these organs with saline solution and isooctane,[3] followed by determination of the ultraviolet absorption maxima of the measured isooctane extracts. The absorption was read in 0.5 cm quartz cells from 260 to 220 mμ on the Cary recording spectrophotometer. Background was negligible, but could be corrected by subtracting the absorption at 260 mμ. In each case the optical densities found at 230.7 mμ were transformed to milligrams of oil by means of a suitable factor found for each new mixture.

RESULTS

1. Emulsion formation

Mice starved 24 hours were given 70 mg of oil composed of methyl oleate and various percentages of corn oil (or, in one case, monopalmitin). The emulsions formed in the small intestines were compared with each other and with that formed from corn oil alone. The averages of the results from these experiments are recorded in table 1, in which the turbidities of the emulsions formed from the oil mixtures are compared, using as the unit standard the washings from the intestines of control animals receiving no oil. These results were reproducible within 5%.

As can be seen from table 1, the intestinal washings from animals receiving methyl oleate-corn oil mixtures were considerably more turbid than those formed from methyl oleate alone. This turbidity is apparently independent of the concentration of corn oil within the range used. The lower value

[3] For a more detailed treatment of this procedure see Mead, Decker and Bennett, J. Nutrition, *43:* 485, 1951.

found using monopalmitin instead of corn oil may have been due to the low solubility of this substance in methyl oleate at body temperature. The higher turbidity of the emulsions formed from 100% corn oil cannot be explained at the present time. In examination of the washings, it could always be seen that those formed from oil mixtures were definitely turbid and contained no large droplets, while those formed from methyl oleate alone were invariably clear and contained many droplets which were filtered out by the glass wool.

TABLE 1

Comparison of the turbidity of emulsions from the small intestines of mice fed mixtures of methyl oleate and corn oil in various proportions

CORN OIL	NO. OF DETERMINATIONS	AVERAGE TURBIDITY
%		
Control	10	1.0 [1]
0	7	1.0
2	3	3.5
5	3	3.5
10	3	3.2
15	2	3.1
100	7	6.9
5% Monopalmitin [2]	1	2.1

[1] Taken as standard.

[2] Fed at 60°. The actual solubility of monopalmitin in methyl oleate at body temperature is much less than this.

2. *Absorption experiments*

Although a complete study has not been carried out on the effect of starvation on the absorption of esters, several experiments have been performed in order to amplify the results reported above.

Quantitative absorption experiments showed the average amount of methyl oleate absorbed in one hour by 7 fasted mice to be 20 ± 2 mg. Incorporation of 10% of corn oil into the methyl oleate brought the amount absorbed to 39 mg (average of three experiments). Normal absorption for this time is 31 ± 8 mg.

An experiment summing up the data reported above and relating emulsion formation and absorption was carried out in the following manner. Two fasted mice were fed about 50 mg of either the methyl oleate-methyl octadecadienoate mixture or the mixture with 10% corn oil added. After one hour the animals were sacrificed, and the stomach and large intestines were washed out with sodium chloride solution and isooctane. The small intestines were first washed out with distilled water as described above for measurement of turbidity, and then with isooctane. The distilled water washings were typical in appearance of those described above for the methyl oleate mixtures. They were filtered and centrifuged, and the turbidities were measured. Isooctane extracts were then made

TABLE 2

The relationship of emulsion formation to fatty ester absorption

MOUSE NO.	AMT.	CORN OIL	OIL RECOVERED IN VARIOUS PORTIONS OF G. I. TRACT					ABSORBED
				Small intestine				
			Stomach	Isooctane extract of intestine	Extract of emulsion	Extract of residues	Large intestine	
	mg	%	%	%	%	%	%	%
1	51.2	0	4.3	0	2.0	37.1	0	56.6
2	59.1	10	12.5	0	11.2	2.4	0	73.8

of the emulsions and of the residues formed during filtration and centrifugation. As can be seen in table 2, corn oil promotes both the emulsification and absorption of methyl oleate in the fasted animal.

The effect of dietary substances other than fat on the absorption of fatty esters was next ascertained. Three mice which had been on the standard fat-free diet (Decker, Fillerup and Mead, '50) for three days were given 50 mg of the methyl oleate-methyl octadecadienoate mixture, sacrificed after one hour and treated as usual. Absorption was found to be normal for this period (37 mg average). No emulsion studies could be done in these experiments because of the presence of large amounts of food in the intestinal washings.

SUMMARY AND CONCLUSIONS

The foregoing experiments have emphasized several facts quite clearly.

The methyl esters of the higher fatty acids are not emulsified to any extent in the intestine of the fasted mouse. The addition of small amounts of corn oil (triglyceride) or monopalmitin (a monoglyceride) promotes the emulsification.

The intestinal absorption of fatty esters has been found to be subnormal in the fasted mouse and to be brought to normality by the addition of corn oil. It has also been found to be normal in the animal on a fat-free diet, indicating that substances in the diet other than glycerides may promote emulsification.

Emulsion formation and absorption have been more closely correlated in an experiment in which both types of information are given for the same animal. Emulsion formation is evidently necessary for optimum absorption.

The data do not necessarily support Frazer's theory of particulate absorption as opposed to the lipolytic theory propounded by Verzar ('48), since emulsification would also favor the action of lipase. However, it can now be stated with more confidence that glycerides are important factors in the emulsification and absorption of fatty substances *in vivo*.

Several results from the literature may also lend themselves to further clarification. Among these are the papers of Barnes, Miller, and Burr ('41a, b, c) who found the rate of absorption of the methyl esters of the conjugated fatty acids of corn oil and that of corn oil itself to be different. The same authors found fat absorption in essential fatty acid-deficient and adrenalectomized animals to be normal. However, in each experiment, methyl esters were administered to animals which had been fasted 18 hours. It is possible that in all cases both the controls and the experimental animals were not carrying on normal fat absorption.

Finally, although Frank (1898) found complete hydrolysis of esters, this may have been because adequate emulsification

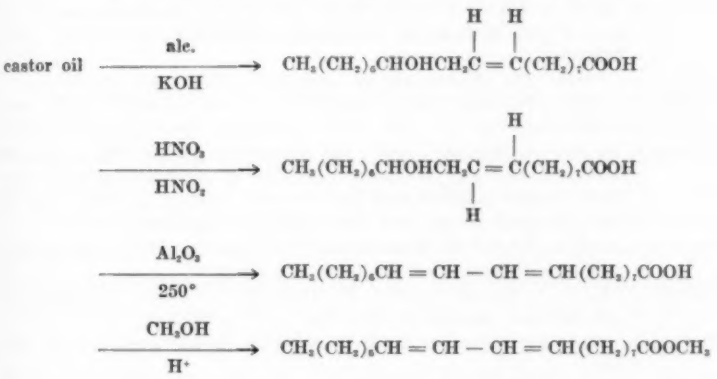
could not occur and absorption had taken the alternate pathway proposed by Frazer (hydrolysis, followed by absorption into the portal system).

Further experiments along these lines are in progress and may serve to elucidate the general mechanism of fat absorption more completely.

APPENDIX

The preparation of methyl 9, 11-octadecadienoate

This ester was prepared acording to the following equations:



9, 11-Octadecadienoic acid was prepared from ricinelaidic acid by a modification of the method of Boeseken and Hoevers ('30). A mixture of 58 gm of recrystallized ricinelaidic acid with 2 gm of Al_2O_3 (Alorco 200 mesh) was heated in a 300 ml two-neck flask while the pressure was slowly reduced to 10 to 20 mm and the temperature allowed to rise to 250–270°. After most of the water had been driven off, a light yellow oil was collected in the receiver. This oil (approximately 15 gm) was dissolved in 20 ml of 80% ethanol with slight warming. After standing for 12 hours at 4°C., colorless needles had been deposited. These were filtered off, washed with ice-cold 70% ethanol and dried *in vacuo;* yield 3.3 gm. The absorption spectrum of this product was taken from 260 to 210 mμ in isooctane, using the Cary recording spectrophotometer. At 230.7 mμ a maximum was obtained which had an $E^{1\%}$ of 879. This extinction value, as will be seen below, shows the product to be approximately 80% pure. After two further crystallizations from 80% ethanol, the compound was obtained as colorless needles, m.p. 51.6 to 52.2°, $E^{1\%}$ (isooctane) 1118 at 230.7 mμ.

Methyl 9, 11-octadecadienoate. Although the preparation of this ester has not been described, the methyl ester was prepared by the method of Boeseken et al.,

('27). One gram of the purified octadecadienoic acid was refluxed with 5 ml of 1 *N* methanolic hydrogen chloride under a stream of nitrogen for two hours. At the end of this period 10 ml of water were added and the resulting suspension was extracted with three 10-ml portions of petroleum ether (b.p. 28 to 30°). The petroleum ether solution was washed with two 10-ml portions of 5% sodium carbonate and one portion of water and dried over anhydrous magnesium sulfate. Evaporation of the solvent with nitrogen at 0°C. yielded about 1 gm of a light yellow oil which solidified in the ice box. This was crystallized from methanol to give 0.8 gm of colorless needles of m.p. 13° and $E^{1\%}$ (isooctane) 1030 at 230.7 mμ. The latter value corresponds to about 97% purity, based on the $E^{1\%}$ value for the acid.

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THE EFFECT OF X-IRRADIATION UPON FAT ABSORPTION IN THE MOUSE[1]

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ONE FIGURE

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The literature from 1920 until the present contains many references which indicate that one of the major effects of whole body x-irradiation is injury to the small intestine (Dennis, Martin and Aldrich, '20; Warren and Whipple, '22).

Martin and Rogers ('23), using dogs, found that irradiation of intestinal loops with a dose which gave only mild skin erythema produced extensive damage of the intestinal mucosa. The same authors ('24) stated that one week after irradiation the absorption of cream by dogs was impaired, as evidenced by lack of fat droplets in the lacteals three hours after ingestion. Since then several groups of workers have found indications of post-irradiation impairment of intestinal absorption.

Dodds and Webster ('24) described altered fat absorption in human leukemia following irradiation. Buchwald ('31), using the technique of Cori, found diminished glucose absorption in rats 20 and 40 hours following a sublethal dose of x-rays. This observation was confirmed by Barron, Wolkowitz and Muntz ('47), who found impaired "permeability" of the intestine within 4 hours after irradiation. A linear relationship was found between the glucose absorption and the

[1] This paper is based on work performed under Contract AT-04-1-GEN-12 between the Atomic Energy Commission and the University of California at Los Angeles.

amount of irradiation, as measured by the amount of glucose remaining in the entire gastrointestinal tract.

The subject has been reviewed by Friedman ('42), with the conclusion that the absorption of sugars and fats is decreased following x-irradiation.

The first indication that the absorbing ability of the intestine may not be impaired by x-irradiation came from the work of Bennett, Bennett, Shaver and Grachus ('50), who found that the absorption of vitamin A was normal or increased after the first day following irradiation of rats with 625 *r*.

With these results in mind, it seemed desirable to make a thorough study of the subject in order that the true state of affairs might be ascertained and the results already reported evaluated. It was decided to make a preliminary study of the effect of x-irradiation on fecal fat and to determine whether any increase in the excretion of fat after irradiation might indicate a lowered absorption.

Following these experiments the absorption of fat by normal mice was determined, using as a tracer the spectrophotometrically determinable methyl ester of 9, 11-octadecadienoic acid. A similar technique has been described by Miller, Barnes, Kass and Burr ('39) using the methyl esters of the conjugated fatty acids derived from corn oil. In the present procedure, the rate of absorption of methyl oleate by the normal mouse was first determined. An appropriate proportion of methyl octadecadienoate was then added to the methyl oleate and the absorption of the mixture was determined. In this way it was proved that the mixture was absorbed as a whole and at the same rate as pure methyl oleate.

That the methyl esters of the fatty acids are not true fats was recognized early in the research. In fact, since they could not by hydrolysis furnish monoglycerides, which, according to Frazer ('48) are necessary for emulsification and absorption of fat in the small intestine, some doubt was held as to whether these compounds could be absorbed normally. That they do indeed follow the usual pathway for triglyceride absorption was shown in a series of experiments (Mead,

Bennett, Decker and Schoenberg ('51) in which it was found that in the fasted animal, emulsion formation and normal absorption of the esters occurred only if glycerides (or certain other substances) were fed simultaneously. Normal absorption was thus maintained by allowing constant access to food to all experimental animals, and thus avoiding the abnormalities brought about by starvation.

EXPERIMENTAL

Materials

The mice used were mature albino females of the CF_1 strain raised in the project animal colony and averaging 27 gm in weight. Methyl oleate was obtained from the Hormel Foundation. Methyl octadecadienoate was synthesized in this laboratory as described elsewhere (Mead, Bennett, Decker and Schoenberg, '51).

Methods

I. Determination of fecal fat. Fecal fat was determined by a modification of the procedure used with human beings by Van de Kamer, ten Bokkel Huinink and Weyers ('49). To 1 gm of feces in a 50-ml Erlenmeyer flask were added 2 ml of 33% potassium hydroxide and 8 ml of 96% ethyl alcohol. The solid material was mashed with a glass rod, and the mixture was then refluxed for 20 minutes. To the cooled mixture were added 3.5 ml of *N* sulfuric acid, followed by 10.0 ml of petroleum ether (b.p. 60 to 70°). The mixture was shaken and centrifuged and most of the petroleum ether layer was pipetted into a conical flask and dried over magnesium sulfate. A 5.0-ml portion of the dried petroleum ether fraction was pipetted off and evaporated. The residue was then titrated in 96% ethyl alcohol with 0.1 *N* sodium hydroxide, using phenolphthalein as an indicator. From the value thus obtained, the amount of fatty acid originally present was calculated.

Feces were allowed to stand 12 hours before analysis, since it was found that very little weight loss occurred after this time, even in a vacuum desiccator. The loss of weight aver-

aged 36% for this period; hence the results, which were calculated on the basis of the dry weight, could be transformed into per cent of wet weight if desirable.

II. Measurement of fat absorption. The determination of the amount of oil absorbed was carried out in two ways, depending on the type of oil involved. In both cases the initial procedure followed a modification of that employed by Irwin, Steenbock and Templin ('36) for use with rats.

The animal to be used was kept on a standard diet until time for the feeding. The required amount of oil (40 to 70 mg) was then delivered well into the oesophagus from a weighed tuberculin syringe. After the required period of time had elapsed, the animal was sacrificed with chloroform and the entire gastrointestinal tract was removed and divided into stomach, small intestine and large intestine, and each section was analyzed separately. The contents of each section were washed out with about 15 ml of 0.85% sodium chloride solution, followed, in the first method, by about 15 ml of petroleum ether (b.p. 28 to 30°) or, in the second, by an equal volume of isooctane.[2] In the case of the stomach, a slit was made in the fundus during the latter stages of the washing, and the organ was finally cut into small pieces and analyzed with the wash liquids. The caecum also was slit during the final stages of the washing but was not cut up or included in the washings. The washings were then shaken for 5 minutes and, after centrifugation to separate the phases, the organic layer was pipetted off as completely as possible. The aqueous layer was washed with 5 ml of the appropriate organic solvent and was then acidified with 9 *N* sulfuric acid and washed with a further 5-ml portion.

In the first method, the petroleum ether solution was dried over magnesium sulfate and evaporated to dryness. The residue was then analyzed for fat by saponification and titration of the fatty acids as described above for fetal fat. This method was used to determine the rate of absorption of pure methyl

[2] Phillips Spectro Grade.

oleate. Two determinations of this type were made for each point and were found to lie on the curve for normal absorption.

In the usual determination, however, a far simpler procedure was employed. The oil used consisted of methyl oleate containing 10% of methyl 9, 11-octadecadienoate, which can be quantitatively determined by measurement of the intensity of its ultraviolet absorption maximum at 230.7 mμ. In carrying out the analysis, isooctane was used instead of petroleum ether because of the superior ultraviolet transparency of the former. Since a determination of the effect of Rockland rat diet on the light absorption characteristics of isooctane extracts of the mouse gastrointestinal tract showed this effect to be negligible, it was thought that the most nearly normal fat absorption could be obtained without preliminary starvation, change of diet or other disturbing influences; hence, the mice were removed from the cage only at the time of injection of the oil and were replaced in the cage until the required time had elapsed. Food was kept in the cages at all times, and was generally found in the stomachs of all animals used. By several determinations made on mice on a fat-free diet, the effect of the very small amounts of fat in the stomach was found to be negligible.

Irradiation of all mice was done at a distance of 54 cm from the x-ray tube, and at a dose rate of 33*r*/minute radiation of the whole body. A 250-KV machine operated at 15 milliamperes was used. The H.V.L. was 185 mm copper. The filters were 0.5 mm copper parabolic, 1.0 mm aluminum, and 0.21 mm copper inherent.

RESULTS

I. Fecal fat

In the determination of fecal fat, 15 mice were divided into three equal groups: group I on a Rockland rat diet; group II on a fat-free diet; and group III on a fat-free diet with corn oil ad libitum. The per cent of fat normally present in the feces of these groups was found in 10, 8 and 5 determinations,

respectively, on pooled samples from each cage. This amount was quite constant for the animals on the fat-free or Rockland diets and probably represents excreted fat rather than unabsorbed dietary material (Sperry and Bloor, '24). The results with the animals on the corn oil supplement, however, were quite variable although in the same general range as the others. Thse results are given in table 1.

All three groups were irradiated with 500 *r*, which is about the midlethal dose for these animals in this laboratory. Fecal fat determinations were then made each day post-irradiation until the 5th day, when the number of animals had been re-

TABLE 1

The per cent fat in the feces of irradiated and non-irradiated mice on various diets

RADIATION (DAYS PRE- OR POST-500 *r*)	FECAL FAT		
	Rockland rat	Fat-free + oil	Fat-free
	%	%	%
Pre	3.08 ± 0.07	4.3 ± 0.5	2.02 ± 0.03
1 day post (single determination)	2.20	4.08	5.17
2 days post (single determination)	2.74	3.66	5.56
4 days post (single determination)	...	5.78	3.27
5 days post (single determination)	2.22	4.97	2.50

duced to only a few survivors and the fecal fat appeared to be approaching the normal value. The general condition of these animals has been described in a previous communication (Decker, Fillerup and Mead, '50). From the results shown in table 1, it can be seen that only in the case of the animals on a fat-free diet was a significant change obtained. This increase in the per cent of fecal fat immediately after irradiation is all the more significant because of the constancy of the normal amount and the fact that no fat was received in the diet. It is possible that it is due in part to desquamation of the intestinal mucosa.

II. Fat absorption

First to be investigated was the absorption of fat by the normal mouse. Preliminary investigations indicated that the mouse is capable of absorbing about 60 mg of oil when given in a single dose. The maximum time for the absorption of a dose of this size appears to be about two hours. After this period, only residual amounts usually remain in the stomach and small intestine, the excess over 60 mg having passed beyond the region of absorption. Some dependence of the total amount absorbed on the weights or body surfaces (Deuel, Hallman and Leonard, '40) of the animals was noted. However, in the case of mice, the differences in weights were too small to warrant a presentation of the data on this basis.

With these facts in mind, absorption was studied at half hour intervals from zero to two hours, and the dose given was slightly more than the total which could be absorbed during the time interval in question.

Several two-minute runs showed recoveries of 94 to 100%, with a maximum loss of 2 to 4 mg of the oil. The other gross features of the normal absorption curve are shown in figure 1, in which the amount of oil absorbed is plotted against time. The range of the points and the standard errors of the means as shown in table 2 indicate some variability in the results. It can readily be seen, however, that a tendency exists for the absorption to take place at a constant rate after the first half-hour. This is not in complete agreement with the data of Barnes, Miller and Burr ('41), who found the rate of absorption of the isomerized methyl esters of the acids derived from corn oil to be dependent upon the concentration of the esters. However, the present data agree with those of most other workers in the field (Deuel, Hallman and Leonard, '40); i.e., that fat is absorbed at a constant rate, independent of the size of the dose, if this is kept within reasonable limits.

The apparently greater absorption during the first half-hour has not been completely explained, but in this case is in agreement with the reported half-hour absorption of the

conjugated methyl esters of corn oil fatty acids by rats (Barnes, Miller and Burr, '41). A few determinations made at the 15- and 45-minute periods showed the same effect and indicated higher absorption than would be expected from the later periods. It is possible that these results may be related

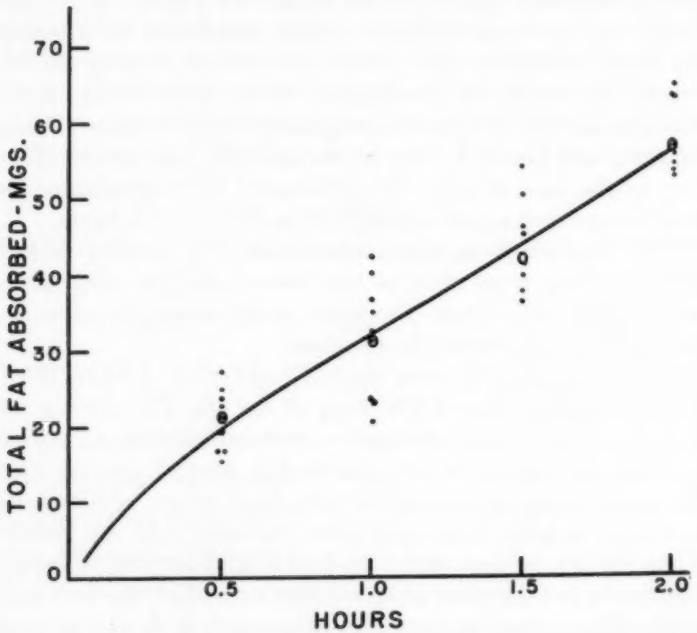

Fig. 1 The rate of absorption of the methyl oleate-methyl octadecadienoate mixture by mice fed normal diet. Circles represent means, while solid dots represent individual values. Each point represents a single determination.

to the "loading phenomenon" described by Frazer ('48), or some other saturation mechanism.

As can be seen from table 2, at the half-hour period an average of 11 mg of oil remains in the stomach; at the one-hour period this has fallen to 8 mg; after the 1.5-hour period an average of 5 mg remains. This residual amount is appar-

ently removed only slowly and some remains in the stomach even after 2.5 hours.

The amounts of oil in the small intestine are high (20 to 30 mg) during the first hour of the absorption period and then taper off to a small residue at two hours.

The amounts found in the large intestine and caecum are usually zero for the first hour and then increase depending on the amount given.

TABLE 2

Average amounts (in mg) recovered from various portions of the gastrointestinal tract at various intervals after dosage and at different times post-irradiation

DURATION OF TEST	TREATMENT OF ANIMAL	AMOUNT ADMIN.	AMOUNT RECOVERED			TOTAL RECOVERY	TOTAL ABSORPTION
			Stomach	Small intestine	Large intestine		
hours							
0.5							
	Normal	62	11	29	1	41	21 ± 4
	2–4 hours post-irrad.	41	25	6	1	32	9
	24 hours post-irrad.	47	17	9	0	26	21
	96 hours post-irrad.	49	20	11	0	31	18
1.0							
	Normal	57	8	17	1	26	31 ± 8
	2–4 hours post-irrad.	46	18	4	1	23	23
	24 hours post-irrad.	48	12	5	2	19	28
	96 hours post-irrad.	47	11	8	1	20	27
1.5							
	Normal	58	5	5	6	16	42 ± 8
	2–4 hours post-irrad.	69	23	6	8	37	32
	24 hours post-irrad.	66	10	5	13	28	38
	96 hours post-irrad.	65	10	7	3	20	45
2.0							
	Normal	75	7	3	8	18	57 ± 6
	2–4 hours post-irrad.	64	12	2	10	24	40
	24 hours post-irrad.	61	21	2	4	27	34
	96 hours post-irrad.	66	18	4	4	26	40

These data, as will be seen below, are important in the interpretation of the effects of irradiation.

For the irradiation experiments groups of 8 mice were given 600 or 700 r [3] whole body x-irradiation, which is considerably above the midlethal dose for mice in this laboratory. The animals were given the doses of oil in pairs at periods of two, 24 and 96 hours post-irradiation. The absorption of the oil was then determined in each pair at either the half-hour and 1.5-hour intervals or at the one- and two-hour intervals. As can be seen from tables 2 and 3, several significant differences exist between the data found for the controls and those found for the irradiated animals.

The first point to be noticed is the great variability of the data found in table 3. Some of the animals were apparently quite normal in absorption, while others showed extreme differences. For this reason, interpretation of the more apparent results of table 2 should be done only cautiously and with an eye to their derivation from the data in table 3.

The two-hour post-irradiation period showed the effects to the greatest extent, followed by the 24- and 96-hour periods in that order.

For the two-hour period the average amounts of oil absorbed were somewhat lower than normal, particularly for the half-hour absorption. From a glance at the tables, it can be seen that this observed decreased absorption was the result of two effects. In the half- and one-hour absorption period, a high percentage of the dose remained in the stomach, while in the later times, although the amounts in the stomach were still high, a large proportion of the oil administered had already appeared in the large intestine, even though the administered dose was not much greater than the maximum absorbable dose (60 mg) and the amount absorbed was subnormal. In some cases, especially in the earlier absorption periods, there was practically no absorption, and the entire dose was recovered from the stomach.

[3] No differences were noted in the effects of the different amounts, possibly because this dose is close to the LD 100.

TABLE 3

The amounts (in mg) of oil recovered from various portions of the gastrointestinal tract at various intervals after dosage and at different times post-irradiation

DURATION OF TEST	AMOUNT ADMIN.	AMOUNT RECOVERED			TOTAL ABSORPTION
		Stomach	Small intestine	Large intestine	
hours	*mg*				
		A. 2 hours post-irradiation			
0.5	29.0	16.5	4.2	0	8.3
	50.0	3.9	19.9	3.9	22.3
	44.5	43.0	0	0	1.5
	41.5	34.7	1.1	0	5.7
1.0	34.6	7.1	6.6	0	20.9
	40.8	7.0	6.6	0	27.2
	56.2	45.8	1.8	0.9	7.7
	51.5	11.5	2.1	1.0	36.9
1.5	82.6	20.0	11.4	0	51.2
	65.8	29.2	2.2	5.9	28.5
	59.8	3.5	.8	25.8	29.7
	69.2	28.8	9.5	0.8	30.1
2.0	49.0	1.4	1.1	12.1	34.4
	66.7	3.8	1.9	22.9	38.1
	61.8	10.8	1.4	3.3	46.3
	77.0	31.3	1.4	0	44.3
		B. 24 hours post-irradiation			
0.5	52.9	13.2	9.5	0	30.2
	43.0	5.4	14.0	0	23.6
	48.3	43.0	0	0	5.3
	43.9	6.5	12.3	0	25.1
1.0	44.6	14.2	4.5	0	25.9
	33.5	2.7	0.9	2.6	27.3
	58.9	18.9	11.8	5.9	22.3
	54.0	10.8	4.4	0	38.8
1.5	68.5	16.4	1.7	0	50.4
	61.4	15.4	11.3	7.5	27.2
	61.0	1.6	5.7	4.5	49.2
	71.1	8.2	0.7	41.2	21.0
2.0	57.6	2.7	0.8	11.8	42.3
	52.6	10.7	0.6	1.1	40.2
	66.5	40.6	2.3	3.6	20.0
	65.1	28.4	3.4	0	33.3
		C. 96 hours post-irradiation			
0.5	47.9	9.9	12.1	0	25.9
	46.8	21.4	2.4	0	23.0
	54.6	13.6	20.8	0	20.2
	45.0	33.6	10.3	0	1.1
1.0	51.4	22.7	7.2	0	21.5
	38.7	4.4	6.9	2.6	24.8
	41.4	7.4	9.0	0	25.0
	57.9	9.6	6.9	0	41.4
1.5	61.0	4.5	2.6	7.1	46.8
	60.5	1.9	6.1	1.9	50.6
	69.2	14.1	4.0	0	51.1
	70.2	19.8	16.9	2.4	31.1
2.0	61.5	11.1	2.2	7.6	40.6
	64.7	9.6	8.5	1.1	45.5
	61.1	8.1	3.8	6.0	43.2
	72.0	43.5	0	0	28.5

A further significant divergence from the normal may be found by comparing the amounts of oil recovered in the small intestine. These were generally low in the irradiated animals and high in the controls. The most significant comparison is that of the ratios of the amounts recovered from the stomach with those of the amounts recovered from the small intestines. For the controls, this ratio was less than unity for the short absorption periods and slightly greater for the longer times. while for the irradiated animals it was always considerably greater than unity.

For the 24- and 96-hour periods post-irradiation, the results are not so readily susceptible of interpretation. Even though the average amounts absorbed were approximately normal, it can be seen that for these times also the amounts recovered from the stomachs were exceptionally high, while those found in the small intestines were lower than normal. Here, also, several cases may be found in which large amounts of oil were recovered from the large intestine even though absorption was subnormal.

DISCUSSION

The severe histological destruction brought about in the gastrointestinal tract by x-irradiation has led many workers to the conclusion that absorption would be impaired. This idea has been borne out by the few studies which have been carried out on the subject. The present experiments, however, are not in agreement with these ideas, and a reinterpretation of some previous work does not support the contention of lowered absorption.

An appearance of decreased absorption could be brought about by an increase in tone and motility of the gastrointestinal tract. This effect has been noted by several workers (Swann, '24; Conard, '50). In the present case, increased tone of pyloric sphincter may have led to the increased time required for the emptying of the stomach. This effect would give the appearance of lowered absorption if the stomach and small intestine were not analyzed separately. Several of the results in the literature may be interpreted on this basis.

Increased peristaltic movement could account for the rapidity with which the fat, once freed from the stomach, was carried beyond the absorbing surfaces of the small intestine. The general picture presented by many of the animals at dissection bears out these suggestions. In a typical animal two to 24 hours post-irradiation, the stomach is packed with food and in a contracted condition, the small intestine is contracted and almost empty, while the large intestine is flaccid and filled with unformed fecal material. It is not proposed that this condition is specific for radiation sickness and, indeed, it may be brought about by many factors.

Finally, the increase in fecal fat noted in some human patients by Dodds and Webster ('24) after x-irradiation in the abdominal region may not be the result of poor absorption, but may rather stem from the same mechanism which caused a post-irradiation increase in the fecal fat of mice on a fat-free diet.

It can be seen, then, from the data presented above, that the major effects of x-irradiation on fat absorption may be explained by changes in motility. One further fact remains for consideration. The small amounts of fat present in the small intestines of irradiated animals even when the stomach emptied normally argues for an actual increase in the rate of absorption, provided the material reaches the absorbing surfaces in large enough quantities and is allowed to remain for a sufficient time. This point, however, needs further elucidation, perhaps by the use of intestinal loops or other techniques, such as drugs designed to eliminate the motility factor.

SUMMARY

Fat absorption in mice was determined using methyl oleate containing 10% of the spectrophotometrically active methyl octadecadienoate as a tracer. The rate of absorption of this mixture was shown to be nearly constant.

Mice which had received 600 to 700 *r* whole body x-irradiation showed a tendency toward increased tone and motility of the gastrointestinal tract, resulting in retention of oil in

the stomach or rapid passage past the absorbing surface. Despite these changes, fat absorption per se appeared to be about normal.

A post-irradiation increase in fecal fat was shown not to be due to lowered absorption, since it occurred in animals on a fat-free diet.

It is concluded that there is no evidence of post-irradiation impairment of fat absorption.

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DRIED WHEY AS A SOURCE OF UNIDENTIFIED FACTORS FOR THE GROWING CHICK[1]

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FOUR FIGURES

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Recent reports indicate that the animal protein factor (APF) complex is composed of unidentified factors in addition to vitamin B_{12}, and that such unidentified factors are required for maximum chick growth (Carlson et al., '49; Menge, Combs and Shorb, '49; Stokstad et al., '49; Combs and coworkers, '50; Sunde et al., '50). Earlier studies by Berry et al. ('43), Hill et al. ('44) and Hill ('48) had shown that dried whey or whey solubles, or both, were possible sources of unidentified chick growth factors, before the isolation of vitamin B_{12} by Rickes et al. ('48). Hill ('48) designated one such growth factor as the "whey factor" and stated further that this nutrient was present in varying amounts in soybean oil meal.

Stokstad and Jukes ('50) and Whitehill et al. ('50) reported that fermentation products of *Streptomyces aureofaciens* — aureomycin, streptomycin, penicillin and sulfasuxidine — promoted the growth of chicks adequately supplied with vitamin B_{12}. Reed and Couch ('50) failed to obtain an increase in the

[1] Animal protein factor concentrate 3 was supplied by Dr. D. F. Green, Merck and Company, Rahway, N. J. Animal protein factor concentrate 4 was supplied by Dr. T. H. Jukes, Lederle Laboratories, Pearl River, N. Y. The dried whey and lactose were supplied by Dr. N. F. Rodgers, Western Condensing Company, Appleton, Wisconsin.

growth of chicks kept on sand litter by adding an APF concentrate which contained only vitamin B_{12} to an all-vegetable protein diet, but obtained a very significant increase in chick growth by adding an APF concentrate obtained from *Streptomyces aureofaciens* fermentation which contained B_{12} and aureomycin.

After the completion of the latter study (Reed and Couch, '50) it was deemed desirable to determine whether chick growth could be further stimulated by adding to the diet a source of unidentified factors in the form of dried whey.

EXPERIMENTAL

Data from 4 separate experiments are reported in the present study. The source of chicks and the general experimental procedure were the same as those described earlier by Reed and Couch ('50).

The average biweekly weights of the chicks were obtained by weighing all birds as a group, counting the birds and dividing the total weight by the number of chicks. As the chicks were counted, every 4th chick was weighed individually in grams. The latter weight was used in calculating the analysis of variance data presented later.

The basal diet (R-1) used in these studies contained 30% ground yellow corn, 28.5% ground milo, 35% soybean oil meal, 3% dehydrated alfalfa leaf meal, 2% steamed bone meal and 1.5% limestone flour. In addition, 5 gm manganese sulphate, 18 gm vitamin D concentrate [2] and 30 gm of BY-21 (riboflavin supplement) were added per 100 lb. of ration. The positive control ration (R-2) was the same as the basal (R-1) except that 3% condensed fish solubles and 6% fish meal were substituted for equivalent amounts of soybean oil meal.

Seven groups of 280 birds each were used in the first test reported. The first group was fed the basal diet (R-1) unsupplemented (table 1). The second group received diet R-3 (5% dried whey substituted for an equivalent amount of

[2] Delsterol.

ground milo). Groups 3 and 4 received 0.25% APF 4 (diet R-4) and 0.25% APF 4 plus 5% dried whey (diet R-5), respectively. Groups 5 and 6 were fed 0.05% APF 3 (diet R-6) and 0.05% APF 3 plus 5% dried whey (diet R-7), respectively. Group 7 received the positive control ration (R-2).

Only 5 groups of 280 birds each were utilized for the second experiment. The first group received the basal diet unsupplemented (diet R-1), while the second group was given an injection of 1 μg of crystalline B_{12} per bird per week. All

TABLE 1

Composition of diets used in experiments 1, 2, 3 and 4 [1]

	DIET NUMBER										
	R-3	R-4	R-5	R-6	R-7	R-8	R-9	R-10	R-11	R-12	R-13	R-14
	%											
Dried whey	5		5		5	3			3			3
APF 4		0.25	0.25								0.50	0.50
APF 3				0.05	0.05							
Lactose							3			3		
	mg/kg											
Procaine penicillin (G)								5.5	5.5	5.5		

[1] Diet R-1 contained 30% ground yellow corn, 28% milo, 35% soybean oil meal, 3% dehydrated alfalfa leaf meal, 2% steamed bone meal, 1.5% limestone flour and ½% salt. In addition, 18 gm Delsterol (vitamin D concentrate), 5 gm manganese sulphate and 30 gm BY-21 (riboflavin supplement) were added per 100 lb. of ration. Substitutions in diet R-1 to make diets R-3 to R-14 inclusive were made in place of ground milo. Six per cent of fish meal and 3% fish solubles were substituted for equivalent amounts of soybean oil meal in diet R-1 to make up diet R-2.

injections were made into the pectoral muscle. The third and 4th groups were fed a supplement of 3% dried whey (diet R-8) substituted for an equivalent amount of ground milo and, in addition, each chick in group 4 received a weekly injection of 1 μg crystalline B_{12}. Group 5 was fed diet R-2.

In the third test an effort was made to determine whether the growth-promoting effect of the dried whey might have been due to the favorable effect of the lactose in dried whey on the microflora of the gastrointestinal tract. Seven groups

of 280 birds each were used in the third experiment. The first and second groups were fed the basal ration (diet R-1) and this ration supplemented with 3% dried whey (diet R-8), respectively (table 1). The third group was fed 3% lactose, which was substituted for an equivalent amount of ground milo (diet R-9). The 4th, 5th and 6th groups were fed 5.5 mg of procaine penicillin (G) per kilogram (diet R-10), and in addition groups 5 and 6 were fed 3% dried whey (diet R-11) and 3% lactose (diet R-12), respectively. Group 7 in this test received diet R-2 (6% fish meal and 3% fish solubles).

Five groups of 280 chicks each were used in the 4th experiment to be reported in this paper. This experiment was designed to study the effect of supplementing the basal ration with APF 4 and with 3% dried whey. The first group was fed the basal diet unsupplemented. The second and third groups were fed 3% dried whey (diet R-8) and 0.5% APF 4 (diet R-13), respectively. Group 4 received 3% dried whey and 0.5% APF 4 (diet R-14). The 5th group received diet R-2 (6% fish meal and 3% fish solubles).

The APF 3 was a fuller's earth adsorbate of vitamin B_{12} [3] and was standardized to contain approximately 27.5 μg B_{12} per gram. Animal protein factor concentrate 4,[4] fish meal and fish solubles were found to contain 0.6, 0.13 and 0.6 μg B_{12} per gram, respectively, according to the microbiological assay method of Skeggs et al. ('48) with *Lactobacillus leichmannii 4797* (American Type Culture Collection) as the test organism. Thiomalic acid was used in the medium as a growth factor for *L. leichmannii,* as a reducing agent and for the protection of B_{12} during autoclaving. Acid production was the criterion used to measure the growth of the microorganism.

RESULTS AND DISCUSSION

First experiment

The addition of dried whey (5%) definitely increased the weights of the birds fed diet R-1 either with (diet R-4) or

[3] Supplied by Merck and Co., Rahway, N. J.

[4] Supplied by Lederle Laboratories, Pearl River, N. Y., and stated to contain 2 to 4 mg aureomycin per gram.

without an APF concentrate (diet R-3). When dried whey was substituted for an equivalent amount of ground milo (group 3, fig. 1), an average increase of 157 gm per bird at 10 weeks of age resulted. When such substitution was made in a ration containing 0.25% APF (group 4, fig. 1), an increase in weight of 190 gm per bird was obtained. Attention is also directed to the fact that the birds fed 5% dried whey and 0.25% APF 4 (group 4) weighed more than did those fed fish

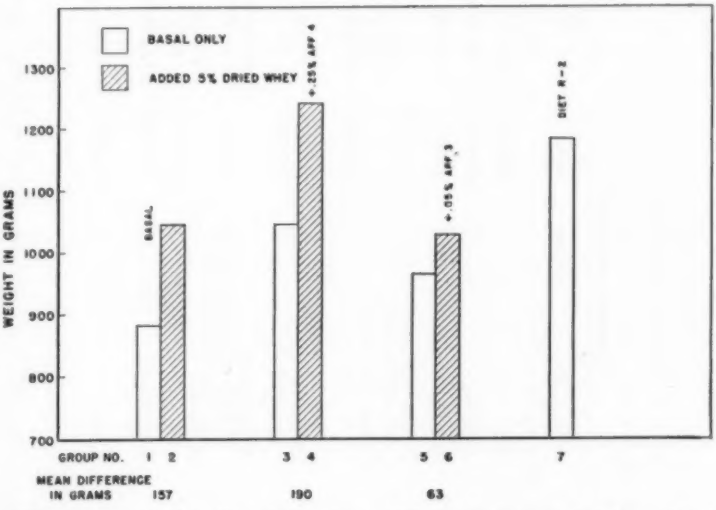

Fig. 1 Effect of feeding dried whey, APF concentrates and fish meal and fish solubles on the weights of New Hampshire chicks at 10 weeks of age.

meal and fish solubles (group 7). In this particular experiment the smallest increase in weight was noted when dried whey was fed with 0.05% APF 3, in which instance an average increase in weight of only 63 gm was noted.

Treatment of these data by analysis of variance (table 2) revealed that there was a significant difference due to treatment and that the groups fed whey (2, 4 and 6, fig. 1) were statistically different from those not fed this supplement

TABLE 2

Analysis of variance

FIRST EXPERIMENT			SECOND EXPERIMENT			FOURTH EXPERIMENT		
Sources of variability	Degrees of freedom	Mean square	Sources of variability	Degrees of freedom	Mean square	Sources of variability	Degrees of freedom	Mean square
Sex	1	5559407 [1]	Sex	1	3339485 [1]	Sex	1	4028719 [1]
Rations	6	664348 [1]	Rations	4	1399416 [1]	Rations	1	1735656 [1]
R-2 vs. others	1	194208	R-2 vs. others	1	3258903 [1]	R-2 vs. others	1	493984 [1]
Whey (R-1, R-4 and R-6) vs. no whey (R-3, R-5 and R-7)	1	894156 [1]	Whey (R-1 vs. R-8)	1	2189491 [1]	Whey (R-1 and R-13) vs. no whey (R-8 and R-14)	1	630405 [1]
Supplements (R-1 vs. R-3 and R-5)	2	1269832 [1]	Supplements (injected B_{12})	1	122222 [1]	Supplements (R-14)	1	5461359 [1]
Supplement × whey	2	119031	Supplements × whey	1	27049	Supplements × whey		356874
Rations × sex	6	116218 [1,2]	Rations × sex	4	15765 [2]	Rations × sex	4	56055 [2]
Individuals	476	22154	Individuals	340	23040	Individuals	320	23312
Total	489	42267	Total	349	48233	Total	329	56703

[1] Denotes significance at the 0.05 level.

[2] The interaction, ration × sex, was used as the error term for testing rations and ration supplements.

(1, 3 and 5, fig. 1). Furthermore, it is apparent that the weights of the birds fed 0.25% APF 4 (diet R-4) and 0.05% APF 3 (diet R-6) are statistically different from those of the chicks fed diet R-1. It is realized that the greater portion of the latter difference is due to the feeding of APF 4 (group 3, fig. 1). There is little question but that the feeding of fish meal and fish solubles produced a significant increase in the weight of the birds.

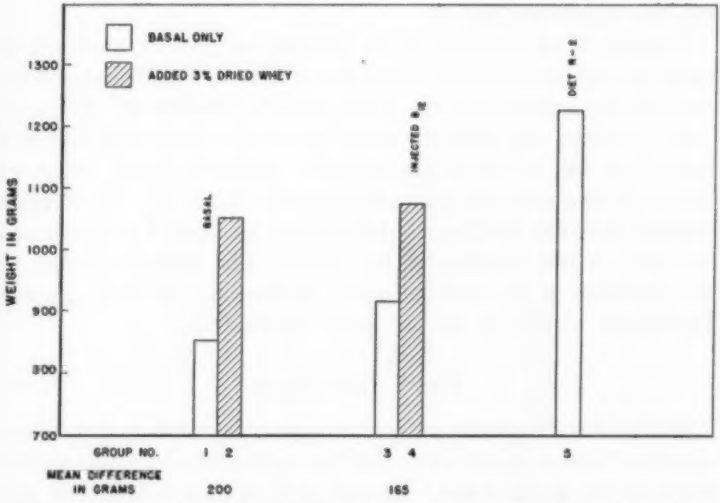

Fig. 2 Effect of feeding dried whey, vitamin B_{12} and fish meal and fish solubles on the weights of New Hampshire chicks at 10 weeks of age.

Second experiment

When dried whey was substituted for an equivalent amount of ground milo in diet R-1, described earlier, the average increase in weight produced was 200 gm per bird (fig. 2). In the third and 4th groups of this test 1 μg B_{12} was injected intramuscularly per bird per week. Such an injection produced a slight increase in the weights of the birds (comparison of groups 1 and 3, fig. 2). However, chicks fed dried

whey without vitamin B_{12} weighed approximately the same as those injected with vitamin B_{12} and fed dried whey (comparison of groups 2 and 4, fig. 2). It may be noted from this test that the feeding of dried whey with injected B_{12} failed to produce birds that weighed as much as did those fed fish meal and fish solubles (comparison of groups 4 and 5, fig. 2). From the above discussion, it is apparent that fish meal and fish solubles supplied unidentified factors which increased the growth of the birds above that noted when B_{12} was injected (fig. 2).

Treatment of the data from the second test by analysis of variance (table 2) shows that there was a significant difference in the weights of the birds due to treatment. There is little question but that the weights of the birds fed diet R-2 (group 5, fig. 2) were statistically different from those of other groups not fed this supplement (table 2). It is quite evident that the feeding of dried whey produced a significant increase in the weights of the birds. The difference due to the injection of B_{12} without whey (groups 1 and 3, fig. 2) was significant although not of great magnitude.

Third experiment

In the third experiment an attempt was made to determine whether lactose might be one of the unidentified growth stimulants in the dried whey. As was true with the first and second experiments, the addition of dried whey to the basal diet produced an increase in the weight of the birds fed this supplement (average of 150 gm). However, the addition of 3% lactose (comparison of groups 1, 2 and 3, fig. 3) did not produce as great an increase as did the dried whey alone. The birds fed penicillin with dried whey (groups 4 and 5) weighed an average of 315 gm more than did those fed the basal ration supplemented with penicillin. The feeding of penicillin with lactose failed to increase the weight of the birds above that obtained when this carbohydrate was fed without penicillin (comparison of groups 3 and 6, fig. 3). It

may also be noted that the feeding of penicillin with dried whey produced birds that weighed almost the same as those fed fish meal and fish solubles (comparison of groups 5 and 7, fig. 3). It may be observed from the results of the third experiment that the unidentified factors found in dried whey apparently have a synergistic activity when fed with an antibiotic such as penicillin. This was particularly apparent when penicillin was fed with 3% dried whey (group 5). The birds

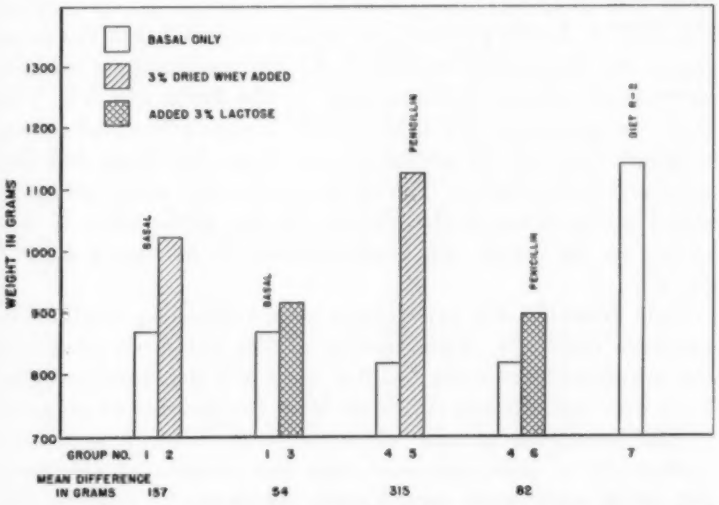

Fig. 3 Effect of supplementing an all-vegetable protein ration with dried whey, lactose, penicillin and fish meal and fish solubles on the weights of New Hampshire chicks at 10 weeks of age.

in the latter group weighed more at the end of the experimental period than did those of any other group, with the exception of the chicks fed fish meal and fish solubles.

Statistical data on the third experiment are not presented, in order to conserve space. These data were treated by the analysis of variance. Such treatment revealed that the feeding of dried whey and fish meal and fish solubles produced significant increases in the weights of the birds. The feeding

of lactose failed to produce a significant increase in the weights of the birds (comparison of groups 1 and 3 and 4 and 6, fig. 3). Chicks fed penicillin (diet R-10, group 4) weighed somewhat less than those fed diet R-1. The synergistic action of whey and penicillin is discussed above.

Fourth experiment

In an earlier experiment Reed and Couch ('50) produced birds that weighed over 1,400 gm at 10 weeks of age by feeding 2% APF 4. In the present test it may be noted that the feeding of 3% dried whey with 0.5% APF 4 produced an average increase of 133 gm in the weight of the birds (fig. 4). The birds in group 4, fed 0.5% APF 4 and 3% dried whey, weighed more at 10 weeks of age than did those fed fish meal and fish solubles. The addition of dried whey also produced an increase in the weights of the birds when it was added to the basal diet (comparison of groups 1 and 2, fig. 4).

Data from the 4th experiment were treated by analysis of variance (table 2). Examination of the latter revealed that the weights of the birds fed fish meal and fish solubles (diet R-2) were statistically different from the weights of all birds in the remaining groups when the latter were grouped together. It is quite apparent that the weights of the birds fed dried whey were significantly increased by feeding this supplement. There is little question but that the feeding of 0.5% APF 4 (diet R-13) produced a significant increase in the weight of the birds over that of those fed diet R-1 unsupplemented.

From the above results it is quite apparent that the feeding of dried whey produced an increase in the weight of the birds in all cases where this supplement was added. Such results are in agreement with the work of Hill et al. ('44) and Hill ('48). Hill ('48) stated that dried whey contained two possible unidentified factors and showed that 4% dried whey in a diet did not furnish significant amounts of amino

acids or B vitamins. Attention is directed to the fact that riboflavin was the only vitamin concentrate added in the present experiments. However, it is not believed on the basis of the results of Hill ('48) that the growth responses obtained in the present study could possibly have been produced by the small quantities of amino acids and B vitamins contributed by the levels of whey used. It is quite apparent that the increase in weight obtained by feeding dried whey

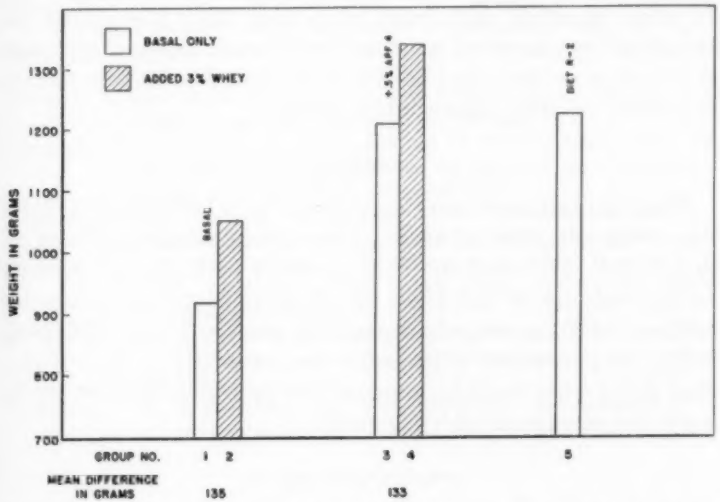

Fig. 4 Effect of adding dried whey, an APF concentrate and fish meal and fish solubles to an all-vegetable protein diet on the weights of New Hampshire chicks at 10 weeks of age.

resulted when only 3% of this product was used (figs. 2, 3 and 4), and therefore it is not necessary to feed a higher level of whey, such as was used in the first experiment. Reed and Couch ('50) have shown earlier that APF 4 produced a greater increase in growth than did APF 3. Such an increase was attributed to the fact that APF 4 was stated by the manufacturer to contain 2 to 4 mg of aureomycin per gram. Results from the present investigation are in agreement with

those of earlier work reported by Carlson et al. ('49); Menge, Combs and Shorb ('49); Stokstad et al. ('49); Combs and co-workers ('50); and Sunde et al. ('50), to the effect that unidentified factors over and above vitamin B_{12} are required for maximum chick growth. Attention is directed particularly to the large number of birds used in the present experiments, and also the fact that the feeding of dried whey produced an increase in the weights of the birds even when they were kept on litter and allowed access to the droppings. It seems possible that dried whey may have stimulated the intestinal synthesis of unidentified growth factors, although it is rather evident that lactose alone failed to stimulate such synthesis to any appreciable extent.

SUMMARY

Four experiments were carried out in which 3 to 5% dried whey was added to an all-vegetable protein (corn-soybean oil meal) diet. The feeding of this product produced an increase in the weights of the birds in all instances, even when fed with an APF concentrate containing aureomycin or with penicillin. In agreement with earlier reports, this work indicates that dried whey contains unidentified factors which are necessary for maximum chick growth.

ACKNOWLEDGMENTS

Delsterol (vitamin D concentrate) was supplied by E. I. DuPont de Nemours and Company, New Brunswick, New Jersey. The BY-21 (riboflavin supplement) was supplied by Commerical Solvents Corporation, Terre Haute, Indiana. The lactose was supplied by the Western Condensing Company, Appleton, Wisconsin. The procaine penicillin (G) was contributed by Merck and Company, Rahway, New Jersey.

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THE UTILIZATION OF INORGANIC SULFATES AND UREA NITROGEN BY LAMBS

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ONE FIGURE

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In experiments in our labortory (Thomas et al., '48, '49, '50; Loosli et al., '49) lambs stored nitrogen and made appreciable gains in weight on purified diets in which urea was practically the sole source of nitrogen. In these studies the 10 amino acids which are known to be dietary essentials for the rat were shown to be synthesized in the rumen of the lamb. Large amounts of methionine were found in the rumen contents and excreta, although the only dietary sulfur was in the form of inorganic sulfates, and thus it appeared that the rumen bacteria were synthesizing this sulfur-containing amino acid from inorganic sulfur and urea nitrogen. In view of the lack of information regarding the utilization of inorganic sulfur by ruminants, the earlier failures to demonstrate benefit from administration of sulfur to sheep (DuToit et al., '36) and rats (Tarver and Schmidt, '42), and the observation that methionine increases the utilization of urea by lambs (Loosli and Harris, '45), it was decided to examine in more detail the sulfur and nitrogen metabolism of sheep fed purified diets.

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EXPERIMENTAL

Three studies were conducted beginning in 1948 to test the comparative responses of lambs fed purified diets containing urea nitrogen with and without added sulfates.

In the first experiment 8 lambs, approximately 9 months of age, were allotted at random into two groups of 4 lambs each. The average weights for the groups were 31 and 29 kg,

TABLE 1

Composition of diets

INGREDIENTS	COMPOSITION OF DIET			
	A	B	C	D
	%	%	%	%
Starch	41.9	41.9	42.9	42.9
Cerelose (Dextrose)	25.0	25.0	25.0	25.0
Cellulose	20.0	20.0	10.0	10.0
Wheat straw	...	...	10.0	10.0
Minerals	5.0 [1]	5.0 [2]	5.0 [1]	5.0 [2]
Lard	4.0	4.0	3.0	3.0
Urea	4.0	4.0	4.0	4.0
Choline chloride	0.1	0.1	0.1	0.1
Vitamins A, D, and E [3]	+	+	+	+

[1] Mineral mixture (grams) — NaCl, 378.0; K_2HPO_4, 668.0; $CaHPO_4 \cdot 2H_2O$, 746.0; $MgSO_4$, 204.0; $CaSO_4 \cdot 2H_2O$, 875.0; $CaCO_3$, 63.3; $Fe(C_6H_5O_7)_2 \cdot 6H_2O$, 58.0; KI, 1.7; $MnSO_4 \cdot 4H_2O$, 3.0; $ZnCl_2$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.7; $CoCl_2$, 0.3; CaF_2, 0.5.

[2] Mineral mixture (grams) — NaCl, 420.0; K_2HPO_4, 742.0; $CaHPO_4 \cdot 2H_2O$, 829.0; $MgCO_3$, 227.0; $CaCO_3$, 610.0; $Fe(C_6H_5O_7) \cdot 6H_2O$, 64.4; KI, 1.9; $MnCl_2 \cdot 4H_2O$, 3.4; $ZnCl_2$, 0.6; $CuCl_2 \cdot 2H_2O$, 0.8; $CoCl_2$, 0.3; CaF_2, 0.6.

[3] Each lamb was fed weekly 1 gm of a vitamin A–D concentrate containing 55,000 I.U. vitamin A and 5,500 I.U. vitamin D per gram and 1.5 gm mixed tocopherols.

respectively. One group was fed the urea-plus-sulfur diet (diet A) and the other the sulfur-deficient diet (diet B) shown in table 1. In this and later studies the lambs were housed in individual pens bedded with wood sawdust. They were fed and watered twice daily.

The lambs did not eat the purified diets readily and they all lost weight during the first 60 days. Thereafter those fed urea-plus-sulfur (diet A) increased in body weight slowly,

while those on the sulfur-deficient diet (diet B) continued to lose weight. The weight changes are summarized in table 2 (Expt. I). Three of the animals on the sulfur-deficient diet died after 128 to 146 days. The other one was changed to a ration of hay and grain. The initial trial was closed after 332 days and the remaining lambs re-allotted to diets A and B for a repeat test. An additional lamb (No. 10) of similar age, previously fed a purified diet containing casein, was also placed on diet A. In this second study (Expt. II, table 2)

TABLE 2

Summary of body weight changes

DIET	EXPERIMENT I			EXPERIMENT II		
	Animal no.	Days on trial	Daily [1] gain	Animal no.	Days on trial	Daily gain
			gm			*gm*
A	1	332	27	1	150	86
Urea plus	2	332	45	3	150	41
sulfur	3	332	27	10	266	82
	4	332	36			
	Ave.		34	Ave.		70
B	5	133 [2]	−68	2	90 [2]	−209
Urea without	6	145	−50	4	100	−145
sulfur	7	128 [2]	−68	6	90 [2]	− 86
	8	146 [2]	−95			
	Ave.		−70	Ave.		−147

[1] Average daily gain or loss excluding first 60 days.

[2] These animals died.

animals fed the sulfur-deficient diet lost weight from the beginning of the trial. Two of them died after 90 days and the other one was transferred to natural feedstuffs. On the urea-plus-sulfur diet the animals all gained in body weight and appeared in good condition after 150 to 266 days on the diet.

A third study was conducted with 8 lambs approximately 8 months of age allotted by chance into two groups and fed diets C and D (table 1). The average initial weight of both

groups was 19 kg. The diets previously used were changed by replacing 10% of the cellulose with wheat straw and by reducing the lard from 4.0 to 3.0% in the hope of increasing palatability and feed intakes. The diet changes appeared helpful, since the lambs fed the sulfur-containing diet gained in weight from the start. The average body weights of the groups of lambs used are shown in figure 1.

The differences in the body weight changes of the animals receiving the sulfur-deficient and the supplemented diets were fully as striking as in the first two tests. After 80 days on

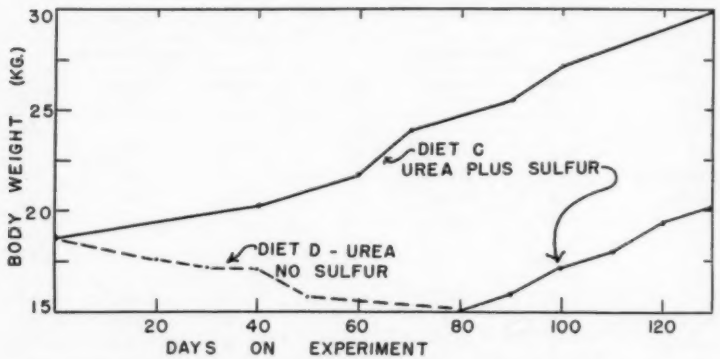

Fig. 1 The average weights of lambs fed the sulfur-deficient and sulfur-supplemented purified diets. Expt. III.

experiment one sulfur-deficient lamb died. Sulfates were added to the diet of the remaining three animals and they began gaining in body weight within a few days. The average daily gain per lamb following sulfate addition was 109 gm, as compared with an average daily weight loss of 50 gm during the previous 75 days on the sulfur-deficient diet. Although the deficient animals generally consumed less feed than the controls after they had been on the diet two months or longer, their energy intakes were sufficient to have maintained weight, at least, had the diet been otherwise adequate. The sulfur-deficient lambs exhibited depraved appetites. They

chewed the wooden pens and pulled and consumed wool from their bodies. Studies of samples of rumen material revealed marked changes in the numbers and types of microflora in the sulfur-deficient animals (Gall et al., '51). Post mortem examination of the animals that died failed to disclose specific pathological lesions.

Sulfur and nitrogen balance data

Sulfur and nitrogen balance tests were conducted from time to time on the animals in the various studies. Collections of feces and urine were made during a 10-day period following a similar period on a constant feed intake. Feed and dried fecal samples were oxidized in a Parr peroxide bomb for total sulfur analyses, the sulfur being measured as barium sulfate. Urine samples were oxidized with the Dennis reagent (Hawk et al., '37) before precipitation of sulfates as the barium salt. Nitrogen was determined by the Kjeldahl procedure. Lambs on the sulfur-deficient diet were always in negative balance for both sulfur and nitrogen. Those fed the urea-plus-sulfur diets were in positive balance consistently. The magnitude of the differences is illustrated by the data in table 3.

Data on wool growth and composition

At the start of the experiments wool was clipped from the backs of the lambs over an area approximately 20 cm square. At intervals during the study wool was again clipped to allow an estimate of the amounts and composition of wool growth. The clipped wool was washed carefully in cold water, then dried and extracted with ethyl ether to obtain the weights of clean wool and samples for analyses. Data obtained from the first study are summarized in table 4.

It is evident that the rate of wool growth was decreased by sulfur deficiency. The differences in wool growth between sulfur-deficient lambs and those fed sulfates became greater as the study progressed. In spite of the marked decrease in the quantity of wool produced, its nitrogen and sulfur con-

tents remained remarkably constant. Barritt and King ('31) have reported that the medullae of wool fibers from black-faced sheep are extremely low in sulfur. In the present study it was not possible to demonstrate a change in the proportion of medulla to cortex and scale between the dietary treatments.

TABLE 3

Sulfur and nitrogen balance data

DIET	ANIMAL NO.	DAILY VALUES FOR			
		Intake	Excretion		Retention
			Feces	Urine	
		gm	gm	gm	gm
Sulfur balances					
Urea plus	5	1.86	0.51	1.04	0.31
sulfur	6	1.86	0.47	1.17	0.22
(C)	7	1.86	0.59	1.09	0.18
	8	1.86	0.60	0.99	0.27
	Mean	1.86	0.54	1.07	0.25
Urea — no	10	0.08	0.02	0.56	—0.50
sulfur	11	0.08	0.03	0.50	—0.45
(D)	12	0.08	0.03	0.39	—0.34
	Mean	0.08	0.03	0.48	—0.43
Nitrogen balances					
Urea plus	5	10.87	3.26	5.16	2.45
sulfur	6	10.87	3.47	6.58	0.82
(C)	7	10.87	2.66	5.90	2.31
	8	10.87	3.47	6.21	1.19
	Mean	10.87	3.22	5.96	1.69
Urea — no	10	2.22	1.40	1.08	—0.26
sulfur	11	2.72	1.25	1.78	—0.31
(D)	12	2.48	1.27	1.45	—0.24
	Mean	2.47 [1]	1.31	1.44	—0.27

[1] The nitrogen intake of these lambs was low because of poor appetites and low food intakes.

Samples of wool were assayed for 11 amino acids. The data, summarized in table 5, show no marked changes in the composition of wool resulting from the sulfur deficiency, although slightly lower values were seen in the case of certain amino acids.

TABLE 4

Influence of dietary sulfur on wool growth and composition

	DIET	
	Added sulfates	Sulfur-deficient
Length of wool fiber		
First 106 days (cm)	3.0	1.8
Last 46 days (cm)	1.5	0.8
Weight of wool		
First 106 days (gm)	30	24
Last 46 days (gm)	10	5
Nitrogen content		
Initial (%)	14.6	15.0
First 106 days (%)	13.4	14.1
Last 46 days (%)	14.2	13.8
Sulfur content		
Initial (%)	3.2	3.2
First 106 days (%)	3.0	3.0
Last 46 days (%)	3.0	2.4

TABLE 5

The amino acid[1] composition of wool

	DIET OF SHEEP		
	Normal[2]	Sulfates added	Sulfur-deficient
	%	%	%
Arginine	8.85	8.57	6.90
Histidine	1.04	1.10	1.15
Isoleucine	3.15	3.20	3.02
Leucine	7.53	7.37	6.12
Lysine	2.79	2.60	2.96
Methionine	0.45	0.46	0.40
Phenylalanine	3.49	3.36	2.80
Threonine	5.09	4.97	4.90
Tryptophan	0.60	0.61	0.54
Tyrosine	4.92	4.50	4.05
Valine	6.20	6.32	5.58
Nitrogen content (%)	14.00	13.99	13.97

[1] Determined microbiologically as described previously (Loosli et al., '49).

[2] Wool clipped from the lambs before they were fed the purified diets.

DISCUSSION

It is somewhat surprising that wool growth continued, even at a retarded rate, for as long as 5 months on a sulfur-deficient diet although the lambs were continuously losing body weight. Thus, the growth of wool would appear to have greater priority on nutrients from the metabolic pool than tissue growth or even maintenance. The observed responses can be explained by assuming that part of the sulfur released by the breakdown of body tissues becomes available for wool growth. Analyses of saliva from the experimental animals after they had been on the sulfur-deficient diet for 75 days or longer revealed sulfur contents varying from 0.5 to 2.2 mg per milliliter. This sulfur would probably be available to the bacteria of the rumen for synthesis of the sulfur-containing amino acids, and would explain a constant finding of some methionine in the rumen contents even after extended periods on a diet very low in sulfur. For example, in experiment II a composite sample of rumen material from lambs on the sulfur-deficient diet taken after 75 days on test contained 0.89 gm of methionine per 16 gm of nitrogen, as compared with 1.29 gm for a similar sample from animals fed the sulfur-supplemented diet.

The data presented show clearly that lambs have a dietary requirement for sulfur. This requirement can be met by inorganic sulfates, at least for the levels of growth achieved in these studies. These results are in agreement with those of Block and Stekol ('50), who showed that radioactive sulfates fed to a dairy cow appeared in the methionine and cystine of its milk.

SUMMARY

Sulfur deficiency was produced in growing lambs fed a purified diet low in sulfur. Deficient lambs exhibited gradual failure of appetite, loss of body weight, emaciation and death. Depraved appetite occurred in all animals. In the absence of dietary sulfur, urea nitrogen was apparently not utilized, since deficient lambs were consistently in negative nitrogen

and sulfur balance. The rate of wool growth decreased but the amino acid composition of the wool produced by sulfur-deficient lambs appeared normal.

ACKNOWLEDGMENT

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THE INFLUENCE OF FOLIC ACID, VITAMIN B_{12}, AND METHYL DONORS ON WHITE BLOOD CELL PRODUCTION IN RATS[1]

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ONE FIGURE

(Received for publication January 5, 1951)

INTRODUCTION

Evidence has recently been presented which indicated that the rat has a requirement for methyl groups in the production of leucocytes (Dinning, Payne and Day, '50). The results suggested that this requirement could be satisfied by methionine alone or by betaine in the presence of vitamin B_{12}; the basal diet contained folic acid. The experiments to be reported in the present paper were designed to study further the methionine requirement for white blood cell formation, the possibility of replacing methionine with other methyl donors, and the influence of folic acid and vitamin B_{12} on the utilization of methyl groups in leucocyte production.

EXPERIMENTAL

Weanling Sprague-Dawley rats of both sexes were housed in individual cages equipped with raised screen floors de-

[1] Research paper No. 952, Journal Series, University of Arkansas. This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service. The folic acid (pteroylglutamic acid) was supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, and the vitamin B_{12} (Rubramin) by E. R. Squibb and Sons, New York.

signed to prevent access to feces. The rats were given the experimental diets described in table 1. Food and water were offered ad libitum and food intake records were kept. The soybean protein contained 1.5% methionine; thus the basal diet supplied 0.27% methionine. The diets were fed for 81 days, after which complete blood counts were made on blood drawn from the tail vein. The data were treated statistically by the analysis of variance method (Snedecor, '46).

RESULTS

Table 2 presents data showing the effects of the supplements on survival, growth rate, and the liver weight-body weight ratio of the rats. Supplementation of the basal diet with both folic acid and vitamin B_{12} or with methionine definitely reduced the mortality. Rats receiving the methionine-deficient diet grew at a very suboptimum rate; growth rates approached normal only in groups receiving additional methionine. The data indicate that, as regards growth, methionine was the limiting factor in the basal diet. Liver weight-body weight ratios were normal only in groups receiving supplements of 0.6% methionine. The elevated ratios in the other groups may indicate that general body growth was more drastically reduced than was hepatic growth.

Peripheral red blood cell counts, hemoglobin concentrations and hematocrit values for all groups were within the normal range for rats and the values are therefore not presented. Data for peripheral white blood cell counts are given in table 3. Rats receiving the basal diet were leucopenic. Supplementation of this diet with folic acid and vitamin B_{12}, or with 0.6% betaine, or with 0.6% choline, resulted in somewhat higher leucocyte counts, although the animals were still leucopenic. However, supplements of 0.6% betaine or choline in the presence of both folic acid and vitamin B_{12} maintained white cell counts at normal levels. Supplementation of the basal diet with methionine, with or without folic acid and vitamin B_{12}, also maintained white cell counts at normal levels. The

TABLE 1

Composition of the experimental diets fed to rats

DIETARY COMPONENT [1]	GROUP NO.									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Soybean protein [2] (gm)	18	18	18	18	18	18	18	18	18	18
Sucrose (gm)	67.9	67.9	67.8	67.3	67.3	67.3	67.3	67.3	67.3	67.3
Crisco (gm)	8	8	8	8	8	8	8	8	8	8
Cod liver oil (gm)	2	2	2	2	2	2	2	2	2	2
Salt mix [3] (gm)	4	4	4	4	4	4	4	4	4	4
DL-methionine (gm)	0	0	0.1	0.6	0	0	0.1	0.6	0	0
Choline chloride (gm)	0.1	0.1	0.1	0.1	0.1	0.7	0.1	0.1	0.1	0.7
Betaine (gm)	0	0	0	0	0.6	0	0	0	0.6	0
Folic acid (mg)	0	0.5	0	0	0	0	0.5	0.5	0.5	0.5
Vitamin B_{12} (Rubramin) (mg)	0	0.005	0	0	0	0	0.005	0.005	0.005	0.005

[1] The following vitamins were added per kilogram to diets for all groups: inositol, 100 mg; thiamine chloride, 15 mg; riboflavin, 5 mg; nicotinamide, 20 mg; calcium pantothenate, 10 mg; pyridoxine hydrochloride, 5 mg; menadione, 0.25 mg; biotin, 0.05 mg.

[2] "Alpha protein," obtained from the Glidden Company, Chicago.

[3] Hubbell et al., J. Nutrition, *14:* 273 (1937).

TABLE 2

The influence of various supplements on survival, growth, and liver weight-body weight ratios of rats receiving a methionine-deficient basal diet

GROUP	SUPPLEMENT	NO. OF RATS	MORTALITY	AVE. DAILY FOOD INTAKE	AVE. DAILY WT. CHANGE	AVE. LIVER WT.
			%	*gm*	*gm*	*% of body wt.*
I	None	12	58	4.9	0.1	6.4
II	Folic acid + B_{12}	8	13	5.6	0.3	5.1
III	0.1% methionine	8	0	10.1	1.1	5.1
IV	0.6% methionine	8	0	11.3	2.5	3.4
V	Betaine	8	13	5.8	0.2	5.9
VI	Choline	8	25	5.8	0.3	5.7
VII	0.1% methionine + folic acid + B_{12}	8	0	9.9	1.5	4.0
VIII	0.6% methionine + folic acid + B_{12}	8	0	12.1	2.7	3.4
IX	Betaine + folic acid + B_{12}	8	0	6.0	0.4	5.3
X	Choline + folic acid + B_{12}	8	0	6.3	0.4	5.3

TABLE 3

The influence of various supplements on peripheral white blood cells of rats (Blood cells reported as thousands per microliter)

METHYL SUPPLEMENT	VITAMIN SUPPLEMENT							
	None				Folic acid + vitamin B_{12}			
	Group no.	Total WBC	Gran.	Lymph.	Group no.	Total WBC	Gran.	Lymph.
None	I	7.2	2.3	4.6	II	14.8	4.3	9.5
0.1% methionine	III	17.4	4.1	12.5	VII	22.3	4.2	16.6
0.6% methionine	IV	19.4	3.0	15.3	VIII	23.1	3.5	18.3
0.6% betaine	V	13.1	4.3	8.0	IX	19.6	4.2	13.9
0.6% choline	VI	12.7	3.7	8.1	X	21.7	4.9	15.2

differences in total leucocyte counts between the various groups were due largely to differences in the numbers of lymphocytes. The analysis of variance data presented in table 4 indicate that the effects of "methyl" treatment or vitamin treatment on peripheral leucocytes were highly significant statistically.

TABLE 4

Analysis of variance for peripheral white blood cell counts of rats receiving the experimental diets

SOURCE OF VARIATION	DEGREE OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	72	2,831.22	
Treatments	9	1,495.32	166.15 [1]
Vitamin treatment	1	609.35	609.35 [1]
"Methyl" treatment	4	739.91	184.98 [1]
Interaction	4	146.05	36.51
Error	63	1,335.90	21.20

[1] $P < 0.01$.

DISCUSSION

Shukers and Day ('43) have shown that inanition may result in a leucopenia in rats. Food intake must therefore be considered in evaluating these experiments. The supplementation of the basal diet with methionine markedly increased food intake. However, the addition of folic acid and vitamin B_{12} to the diet containing additional choline or betaine did not significantly increase food intake but did result in an increased leucocyte count. These observations would indicate that inanition was not playing a major role in producing the observed differences in number of peripheral white blood cells.

The effects of folic acid and vitamin B_{12} on leucocyte production were directly related to the level of choline and betaine in the diet. The data suggest, therefore, that these vitamins function in the utilization of methyl groups from

such compounds. Supplements of methionine without folic acid and vitamin B_{12} maintained white cell counts at normal levels, whereas betaine and choline were effective in this regard only in the presence of folic acid and vitamin B_{12}. This suggests that these vitamins may function in transmethylation reactions resulting in methionine synthesis. Other data

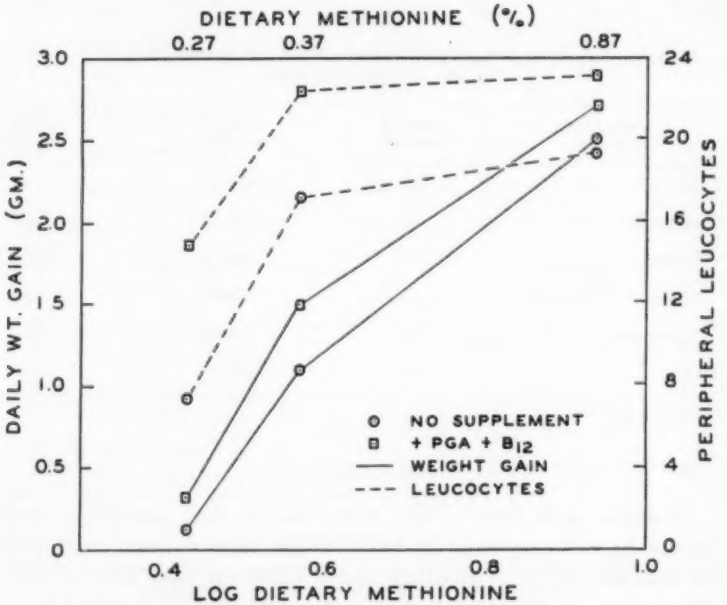

Fig. 1 Growth rates and peripheral white blood cells for rats receiving graded levels of dietary methionine with and without supplements of folic acid and vitamin B_{12}.

from this laboratory (Dinning, Keith and Day, '51) demonstrate that livers from folic acid-deficient chicks exhibit a reduced ability to form methionine from homocysteine and choline or betaine *in vitro*. Oginsky ('50) has reported that dietary vitamin B_{12} increases the rate of methionine formation by rat livers. The failure of suplements of choline or betaine with folic acid and vitamin B_{12} to support growth

may indicate that homocysteine was the limiting factor. The results also suggest that the methionine requirement for leucocyte production is smaller than that for growth, and that when supplied with an inadequate amount of methionine the rat uses it preferentially for leucocyte production. The graphical presentation of the data in figure 1 supports this hypothesis. With increase in dietary methionine the curve for peripheral leucocytes breaks much more sharply than does the growth curve.

The experiments reported in this paper indicate that the rat has a very definite requirement for methyl groups for the production of leucocytes. This requirement may be satisfied by methionine alone, or by choline or betaine in the presence of dietary folic acid and vitamin B_{12}, but not by choline or betaine in the absence of folic acid and vitamin B_{12}.

SUMMARY

Weanling Sprague-Dawley rats were given a basal methionine-deficient, purified diet. Various groups received this diet with supplements of methionine, choline, or betaine, with and without folic acid and vitamin B_{12}. Rats receiving the basal diet alone developed leucopenia. The leucopenia was prevented by supplements of methionine with or without folic acid and vitamin B_{12}, or by choline or betaine with supplements of folic acid and vitamin B_{12}. Supplements of choline or betaine in the absence of added folic acid and vitamin B_{12} failed to prevent leucopenia.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT POLYMERIZATION [1, 2]

I. THE RELATION OF AUTOXIDATION TO DECREASE IN THE NUTRITIONAL VALUE OF HEATED LINSEED OIL

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Ingestion of heat polymerized linseed oil was followed by lowered growth rates in rats, this growth depression becoming more severe with increased lengths of heating time of the oils and with increased amounts of oil in the diet (Crampton et al., '51). Our earlier experiments indicated that the low nutritive value of heated linseed oil diets is attributable to the oil itself and not to the effects of the heated oil upon non-lipid diet constituents.

As thermal treatment causes oil to be less stable to oxidation, it has been proposed that biologically deleterious products may accumulate through progressive autoxidation during storage of the oil-containing diets at room temperature. Oven temperatures used during the baking of the diets may also promote oxidative changes.

The extent of autoxidation can be controlled by the addition of an anti-oxidant. A mixture of nordihydroguararetic acid (NDGA) with citric acid has been shown to be an effective

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[2] Issued as paper No. 260 of the Canadian Committee on Food Preservation.

anti-oxidant for edible fats, increasing stability even in baked products (Mattil et al., '45; Higgins and Black, '45).

The question of vitamin E deficiency in heated oils also seemed worthy of consideration at this time, since polymerization at 275°C. would destroy any of this vitamin inherent in the original oil. The absence of vitamin E, a natural antioxidant, presumably permits a more rapid onset of autoxidation. In addition, a relation of vitamin E to growth, over and above its stabilization of linoleic acid, has been shown in insects by Fraenkel and Blewett ('46). In the feeding of heated lard to rats, Morris and his co-workers ('43) noted a depression of growth and also a paralysis simulating a vitamin E deficiency. Therefore it was possible that in our tests thermal destruction of vitamin E was responsible in part for the adverse effects we had observed to result from feeding heat-polymerized linseed oil.

EXPERIMENTAL

Feeding trials were conducted to examine the effects of baking, of controlled autoxidation, and of vitamin E supplementation of diets containing heat-polymerized linseed oil.

Oil preparation

Polymerization and acetone segregation were performed according to the method of Privett, McFarlane and Gass ('47). Preliminary trials in our laboratories showed no nutritional difference between whole heated oil and its acetone soluble fraction, beyond a decrease in intake of the diets containing the latter. This effect was thought to be due to residual traces of acetone (Langerman, '49). Therefore in the present test, in which acetone segregation of heated oil was involved, the control (unheated) oil was subjected to like treatment with acetone.

Where oils were artificially stabilized (test II), an antioxidant mixture of NDGA with citric acid was added as 0.05% of the oil. Peroxide values on these oils when freshly prepared

and after 28 days of storage at room temperature were estimated by the method of Skellon and Wills ('48). Peroxide determinations on chloroform extracts of the oil-containing diets were made at intervals throughout the 28-day feeding trial by a modification of the Skellon and Wills method (see appendix).

Biological procedure

The animals used in these trials were albino rats ranging in age from 21 to 28 days at the outset of the test period. They received, ad libitum, the diets described in table 1.

TABLE 1

Composition of diets

INGREDIENTS	DIET A (test II)	DIET B (tests I and III)
White flour (%)	47	54
Milk powder (%)	19	19
Casein (%)	11.5	11.5
Oil (%)	20	10
Bone meal (%)	2	2
Salt (%)	0.5	0.5
Yeast (%)	..	3
Vitamins		
Thiamine	10 p.p.m.	..
Niacin	230 p.p.m.	..
Riboflavin	5 p.p.m.	..

Diets minus vitamins or yeast were baked (except where specified in test I) for 20 minutes at 375°F. and the resulting biscuit granulated and air-dried for 24 hours. Vitamin supplements in diet A, or yeast in the case of diet B, were then added. Weekly doses of cod liver oil were administered orally to supply 175 I.U. of vitamin A and 35 I.U. of vitamin D.

In addition to these vitamins, half of the animals in test III each week received orally alpha-tocopherol dissolved in corn oil. The doses, administered two days following the A and D supplementation, were of such amount as to supply 7 mg of vitamin E to each animal.

RESULTS

When the oils were stabilized, there was no appreciable accumulation of easily reducible peroxides. However, definite signs of progressive peroxidation were shown in the unstabilized oil over the 28-day trial. These facts are evident from the peroxide values given in table 2 and calculated at the beginning and the end of the test period on oil extracted from the feed and also on oil that was not combined with the feed.

It is true that peroxide values are not a full indication of rates of total oxygen absorption and that other diet components may have been attacked by peroxides as rapidly as

TABLE 2

Peroxide values

(Calculated as milligrams of peroxide oxygen per kilogram)

	OIL EXTRACTED FROM FEED		STORED OIL	
	Fresh	After 28 days	Fresh	After 28 days
Unheated oil	99	276	47	1,334
Unheated stabilized oil	26	55	49	172
Heated oil	116	333	0	270
Heated stabilized oil	25	38	0	51

these were formed. In such an event, no accumulation of easily reducible peroxides would have been noted. However, a steady increase in peroxide values in unstabilized oils throughout the test points to the likelihood that extensive peroxide decomposition had not yet begun in the stabilized samples.

The growth rates and feed intakes of the animals in test II, given in table 3, show that the stabilized oil was no better nutritionally than the unstabilized control oil. The equality of the growth responses of animals fed heated oils regardless of anti-oxidant treatment makes it unlikely that autoxidation is responsible for the low nutritive value of polymerized linseed oil.

This is further supported by the data from test I, where the growth responses of the animals show that baking did not influence the growth inhibition exerted by the heated oil. Exposure to oven temperatures would, if anything, encourage

TABLE 3

Effect of baking, of anti-oxidant stabilization, and of vitamin E supplementation on the nutritive value of unheated or heated whole linseed oil or on the acetone soluble fraction of the heated oil

TEST NO.	OIL USED	TREATMENT	OIL IN DIET	NO. ANIMALS	AVE. DAILY GAIN	AVE. DAILY FEED INTAKE	GAINS PER 1,000 CAL.[1]
			%		gm	gm	gm
I	Unheated	Diet baked	10	8	4.3	12.1	72
	Unheated	Diet unbaked	10	8	4.2	12.4	69
	Heated	Diet baked	10	8	3.1	10.6	60
	Heated	Diet unbaked	10	8	2.7	9.7	57
II	Unheated	nil	20	10	3.7	10.1	68
	Unheated	Oil stabilized[2]	20	10	3.8	9.6	73
	Heated (acetone soluble fraction)	nil	20	10	—0.1	6.0	..
	Heated (acetone soluble fraction)	Oil stabilized[2]	20	10	—0.1	5.8	..
III	Heated	nil	10	24	2.4	10.0	49
	Heated	Vitamin E supplementation to diet	10	24	2.3	9.8	48

[1] Gross calories (calculated from heats of combustion): 10% fat diet contained 4.9 Cal./gm; 20% fat diet contained 5.4 Cal./gm.

[2] Protected against autoxidation with NDGA and citric acid.

autoxidation. These results imply that the damaging factor in heated oil is not produced oxidatively.

Supplementation with vitamin E failed to alter the nutritive value of polymerized linseed oil (test III).

CONCLUSIONS

The results of these experiments show that peroxidation of polymerized linseed oil is not concerned in the development of any toxic factor, and also suggest that a lack of vitamin E is not involved in the growth inhibition resulting from the ingestion of heated oil.

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Thanks are also due to Miss Barbara Clark for her aid in conducting these feeding tests.

APPENDIX

Modification of the Skellon and Wills procedure for peroxide determinations on oil extracted from diets

A weighed sample of biscuit containing about 1 gm of the oil was shaken up with a 25-ml portion of chloroform and the filtrates were combined. The extract was evaporated to 10 ml by passing over it oxygen-free nitrogen *without heating*, and transferred to a 250-ml flask containing 2 gm sodium bicarbonate. A stopper was fitted, carrying a short piece of glass tubing capped by a rubber Bunsen valve. This served as a one-way pressure relief valve. Fifteen milliliters of glacial acetic acid were used to rinse the last traces of chloroform extract into the reaction flask, and the acid-bicarbonate reaction released CO_2 which swept air out of the flask through the relief valve. When foaming subsided, 1 ml of saturated fresh potassium iodide solution was added, and the tightly stoppered flask was left in the dark for one hour. Fifty milliliters of boiled, cooled water were then added, and the iodine titrated with 0.002 N thiosulfate.

Peroxide values were calculated as milligrams of peroxide oxygen per kilogram of oil, thus:

$$PV = \frac{V \times N \times 16 \times 10^3}{W \times P}$$

where,

V = Volume of thiosulfate used (ml)
N = Normality of thiosulfate
W = Sample weight (gm)
P = Per cent oil in sample.

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THE APPARENT DIGESTIBILITY OF ESSENTIALLY SIMILAR DIETS BY RATS, GUINEA PIGS, SHEEP, SWINE AND BY HUMAN SUBJECTS [1]

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INTRODUCTION

Since they were proposed by Atwater and Bryant, in 1899, the average physiological fuel values of 4, 9, and 4 Cal./gm for protein, fat and carbohydrate, respectively, have been widely, but sometimes not too accurately, used to compute the metabolizable energy of a food or food mixture. Recently a Committee on Caloric Conversion Factors and Food Composition Tables of the United Nations Food and Agriculture Organization ('47) called attention to the limitations of Atwater's average values, and recommended "further research of the energy yielding constituents of food and the derivation of caloric values."

Partly as a consequence of this recommendation a research project was undertaken in this laboratory, designed to obtain data on the source and yield of usable energy from foods; the significance of species of animal with respect to such values; and the agreement between physiological fuel values determined on foods eaten as the entire diet and those obtained for the same food consumed in combination with other foods. The species of animals included human subjects, rats, guinea pigs, sheep and swine.

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada. Journal Series No. 278.

The trials herein reported dealt only with the apparent digestibility of diets composed essentially of wheat and designed to constitute the basal diets to be used with those foods which cannot constitute entire rations.

The trials were carried out in two series, the first of which involved human subjects and the second the 4 other species of animals.

EXPERIMENTAL

Series I

In the preliminary tests the wheat was prepared for feeding by grinding the grain and baking it, with minor admixtures, into loaves. This, however, was found to be costly in time and labor, due partly to the use of yeast as the leavening agent in the bread. It was decided, therefore, to prepare the wheat in the form of muffins. The formula for the muffins, in grams, was: ground wheat 100, sucrose 3, baking powder 4, corn oil 3, salt 1, water 100. This mixture, which made 4 muffins, was baked at 350°F. for 50 minutes.

Our preliminary tests also indicated that some provision for variety in the form of the diet was desirable with the human subjects, and it seemed possible that a commercially prepared baked product, shredded wheat, might prove to have the same energy value as the laboratory-prepared muffins, and thus could be used interchangeably with them. This product would have the added advantage of commercial preparation in the case of sheep and swine, where the quantities needed were relatively large. As an alternative to shredded wheat, cracked wheat porridge seemed to be a possibility.

Series I included, therefore, experiments designed to compare, with human subjects only, three diets, consisting essentially of wheat in the form of baked muffins; as shredded wheat; or as cracked wheat porridge. They were fed to 6 healthy graduate students (three males and three females). Each diet, in addition to the wheat, included sugar or honey or both, butter or corn oil or both, cream, skim milk pow-

der and salt. The muffins were served with honey and butter. The skim milk powder was reconstituted with water and used as a beverage or on the cereal as desired. The cream was used in tea or coffee, or to enrich the skim milk. The shredded wheat was served without alteration. It was eaten with the reconstituted skim milk, the sucrose, the cream and the salt. The porridge was made by cooking the wheat in a double boiler for 90 minutes. It was served with sucrose, cream, and reconstituted skim milk. With all diets tea or coffee was served ad libitum. The dry matter contributed by each of the diet ingredients is shown in table 1.

TABLE 1

Diet mixture, showing dry matter contributed by each ingredient

DIET CONSTITUENTS	% DRY MATTER SUPPLIED BY EACH CONSTITUENT		
	Wheat muffin diet	Shredded wheat diet	Cracked wheat porridge diet
Ground wheat	67	..	..
Shredded wheat	..	68	..
Cracked wheat	..	..	68
Sucrose	2	7	7
Honey	7	..	..
Corn oil	2	..	..
Butter	2	..	..
Cream (35%)	3	11	11
Skim milk powder	13	13	13
Salt	1	1	1
Baking powder	3	..	..

The wheat muffin diet was fed for one 5-day period; the shredded wheat for two 4-day periods; and the cracked wheat porridge for one 4-day period. All were served three times a day in the quantities desired. Carmine markers were used to aid in identifying feces from the test diets.

In addition to proximate principle analyses, using A.O.A.C. methods, the gross energy value of the muffins, shredded wheat and cracked wheat was determined by bomb calorimeter. For the other materials in the diets the composition was taken from the table of food values published by the

Nutrition Division of the Department of National Health and Welfare, Ottawa, Canada ('46), and their gross energy value then calculated from heats of combustion given by Atwater and Bryant (1899).

Observations and results. The cracked wheat porridge diet was not acceptable. The subjects would not eat sufficient of it to allay hunger. Upon examination of the mean daily caloric intakes of each diet, those of the ground wheat and of the shredded wheat were close to estimated maintenance needs and showed no significant differences (2,374 vs. 2,285 Cal.), while that of the cracked wheat diet was significantly lower (1,120 Cal.)

Table 2 shows the mean digestion coefficients obtained with the three diets.

The analysis of variance revealed statistically significant differences between the cracked wheat porridge diet and either one of the other diets in all fractions but ether extract. That there would be no difference in the digestibility of the ether extract was to be expected, since the source of most (85%) of the fat in all three diets was the same (i.e., butterfat). Since the crude fibre fraction was only 1.3% of the total shredded wheat diet, the negative coefficient obtained was probably due to experimental and analytical errors. The higher value obtained for the crude fibre of the cracked wheat porridge diet may have been caused, partly, by the long moist cooking.

The values obtained for total carbohydrates (90, 92 and 91%) were somewhat lower than the values of 97.6 to 99.8% which Sealock et al. reported in 1941. However, in their diets wheat supplied approximately 50% of the carbohydrate, whereas in these diets approximately 80% of the total carbohydrate originated in the wheat, which may in part account for the difference.

The digestion coefficients for energy (90, 89 and 87%), of which carbohydrate was the main source, agree quite closely with the values of 88 and 89% reported by McCance and Walsham ('48) and with those of 86.9 and 87.1% reported by

TABLE 2

Coefficients of apparent digestibility obtained with three "wheat" diets

DIETS	NUMBER OF OBSERVATIONS	DRY MATTER	GROSS ENERGY	PROTEIN	ETHER EXTRACT	TOTAL CARBO.	N-FREE EXTRACT	CRUDE FIBER
		%	%	%	%	%	%	%
Ground wheat muffins	6	90	90	89	84	92	94	7
Shredded wheat	12	89	89	84	84	92	94	—7
Cracked wheat porridge	6	87	87	81	84	91	93	28
Necessary difference to cover chance variation $\left(\frac{s}{\sqrt{n}} \times \sqrt{2} \times t_{.05}\right)$	Shredded wheat vs. either muffins or porridge		2.0	3.9	3.4	0.7	0.8	24.4
	Between muffins and porridge		2.3	4.5	4.0	0.8	0.9	28.3

TABLE 3

Animals involved in third series of experiments

SPECIES	NUMBER AND DESCRIPTION	DAYS ON TEST		METHOD OF FECES SEPARATION	FEEDING PRACTICE	APPROX. AVE. DAILY DRY MATTER INTAKE	SPECIAL SUPPLEMENTS
		Prel.	Coll.				
Rats	14 adult males	6	7	Time collection	ad libitum	28 gm	None
Guinea pigs	12 adult males	6	7	Time collection	ad libitum	30 gm	0.75 mg vit. C. daily
Swine	4 Yorkshire gilts (150-lb. wt.)	4	5	Carmine markers	Weighed allowances twice daily	2.8 lb.	None
Sheep	4 yearling wethers	4	7	Time collection	Weighed allowances twice daily	2.0 lb.	Chopped hay (see text)

Macrae et al. ('42). The test diets of these investigators were similar in ingredients but differed from those used in the present trials in the proportions employed. Wheat provided 75 to 90% of the total calories in their trials and 63 to 69% in ours. Moreover, the digestibility values reported by Macrae were calculated for wheat alone, on the assumption that the other dietary constituents were totally absorbed. Thus his values are somewhat lower than they would be for the total diet.

These two groups of investigators also reported protein digestibility values for diets containing Canadian wheat of 83.3% and 85.7% (Macrae) and 84.9% (McCance and Walsham). These agree fairly well with the values obtained in this experiment, though the proportion of nitrogen provided by wheat was much greater in the Macrae tests (90 to 100%) than in those of McCance and Walsham (65 to 69%).

It seemed evident that wheat muffins as prepared in these studies and commercial shredded wheat could be used interchangeably or in combination as basal diets for human digestion studies.

Series II

This series was comparable to series I but involved the 4 species other than the human subjects. The sugar, honey, cream and butter supplements used in the human tests were replaced equicalorically by cornstarch and corn oil. The ground wheat mixture (see series I) was baked and then finely ground. The shredded wheat diet was prepared by coarsely grinding the commercial biscuits and adding the remaining ingredients. Wheat porridge was not included.

Preliminary tests had indicated that it would be necessary to feed the sheep a small quantity of chopped hay along with the wheat diet in order to maintain normal consumption. Thus for this species the digestibility of the wheat diet was determined indirectly, assuming that the rate of change in digestibility of the complete ration with increasing proportions of concentrate to roughage is a measure of the digesti-

bility of the concentrate (Carbery, Chatterjee and Hye, '34). Three levels of concentrate to one of hay were used.

It had also been found that many guinea pigs were likely to consume the ground wheat combinations reluctantly. Individuals, however, differed in their willingness to eat this type of food, and consequently a total of 18 animals were started on test, 6 of which were eventually discarded because of failure to consume normal quantities of the food. (One of the difficulties with guinea pigs appears to be their dislike of ground feed, for it has since been found that if the same ground mixture is prepared in pellet form it is readily eaten.) For those animals that would consume the ground diet, feces appeared to be normal, and the animals maintained their live weights and otherwise appeared healthy.

Both rats and guinea pigs were fed ad libitum, with accurate feed records kept over the periods during which feces collections were made. Omission of markers in digestion studies employing the ad libitum feeding of diets which do not permit selective consumption of the feed ingredients has been shown in our laboratory to be permissible with swine (Crampton and Bell, '47), and with rats (unpublished data). No direct evidence in the case of guinea pigs is available, but markers obviously cannot be used with this species because of the large functional caecum and the consequent mixing of the ingesta from adjacent meals.

Details concerning the animals involved are shown in table 3.

The mean digestion coefficients obtained are shown in table 4. Included for ease of comparison are comparable data for the human subjects in series I.

The analysis of variance is shown in table 5, from which it is seen that for the same proximate principle [2] there were

[2] The coefficients for crude fibre were highly variable for all species except for the rat. The negative coefficients obtained were obviously false values, and were presumably due to analytical errors arising from the fact that in both wheat diets the crude fibre content was of very small order. Because of this, the carbohydrate fraction of the diets was represented by total carbohydrate in the analysis of variance.

TABLE 4

Coefficients of apparent digestibility of various food fractions of two wheat diets as determined for 5 species

NUTRIENT	DIET	SPECIES					MEAN OF TWO DIETS FOR 5 SPECIES
		Humans [1]	Rats	Guinea pigs	Sheep [2]	Swine	
Dry matter	Ground wheat	90	88	85	79	91	87
	Shredded wheat	89	90	89	80	92	
Gross energy	Ground wheat	90	87	83	75	91	86
	Shredded wheat	89	89	88	80	91	
Crude protein	Ground wheat	89	79	76	76	92	82
	Shredded wheat	84	79	80	76	90	
Ether extract	Ground wheat	84	76	35	90	71	71
	Shredded wheat	84	80	34	85	73	
Total carbohydrate	Ground wheat	92	90	100	87	93	92
	Shredded wheat	92	92	92	89	94	
N-free extract	Ground wheat	94	92	100	89	95	
	Shredded wheat	94	94	93	90	96	
Crude fiber	Ground wheat	7	10	36	—4	15	
	Shredded wheat	—7	11	51	—17	—23	

[1] From series I.

[2] Determined indirectly from hay-wheat mixtures.

no significant differences in digestibility, either among the species or between the two diets.

A significant difference was found among the digestibilities of the different nutrients, as was to be expected. The digestibility of protein was significantly lower than that of total carbohydrate; while ether extract digestion, except for the sheep, was lower than that of any of the other nutrients.

TABLE 5

Analysis of variance of digestion coefficients of 5 nutrients of two wheat diets as determined for 5 species

SOURCE OF VARIATION	D/F	VARIANCE	S.D.	VARIANCE RATIO	
				Observed	Necessary minimum (P = 0.05)
All causes 49	..				
Nutrients	4	622.5		5.6	3.0
Diets	1	3.0		..	..
Species	4	249.8		2.3	3.0
Nutrients × diets	4	4.0		..	
Diets × species	4	3.5		..	
Remainder (error)	32	110.3	3.4	..	

CONCLUSIONS

The results thus far obtained suggest that, of themselves, the anatomical differences in the digestive systems of the 5 species studied do not result in large differences in digestive capacity with respect to diets of negligible crude fibre content.

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DENTAL CARIES IN THE COTTON RAT

XII. NATURAL VERSUS REFINED SUGARS [1]

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INTRODUCTION

The influential role of carbohydrate in the production of dental caries has been known and studied since the early work of W. D. Miller. Numerous nutrition surveys of "primitive" people in their natural habitat and in contact with "civilization" indicate a high degree of correlation between refined foods and caries (American Dental Association, '39). Toverud ('49), in an extensive survey of the dental health of Norwegian children, concluded that an important factor in the decreased dental decay of these children during World War II was the reduced consumption of refined carbohydrates and an increased consumption of natural foodstuffs. Similar observations have been made by Sognnaes ('48), who concluded that the effect was not solely due to a change in oral environment. On the other hand, Waugh and Waugh ('40), who fed natural sugars, represented by maple syrup or honey, to adult Eskimos reported that natural sugars were considerably less cariogenic than refined sugars. These authors

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also concluded on the basis of balance studies of Eskimo children with and without dental caries (Siegel et al., '40) that there appears to be no basis for the view frequently held that the freedom from caries enjoyed by primitive people is due to a nutritional superiority of "natural" as compared to "civilized" diets. This conclusion is in sharp contrast to the results of studies cited above as well as the work of Osborn and co-workers ('37a, b, c), who have studied the relation of diet to caries production in the South African Bantu. These investigators noted the greater destructive effect *in vitro* and *in vivo* of refined sugar and wheat meal as compared to unrefined cane sugar juice and whole wheat, and concluded that this effect may be explained in part by the loss of minerals during the refining process.

Volker and Pinkerton ('47) reported that acid production was similar when the unstimulated saliva of carious people was incubated with dextrose, sucrose or starch, or raw as opposed to refined sugar cane. However, a marked difference in pH changes was noted by Stephan and Hemmens ('47) when several kinds of oral microorganisms were allowed to act on substrates of various carbohydrates. Changes in pH caused by soluble starch were either very slow or were not produced at all, and lactose resulted in considerably slower pH changes than other simple sugars. Thus, although the role of natural and refined carbohydrates in the production of dental caries has been studied at length in humans and animals, the evidence is still contradictory.

Because of the high susceptibility of cotton rats to tooth decay on a purified diet without the presence of fracture particles, this species appeared suitable for the study of the role of natural foodstuffs in the production of dental caries. Schweigert and co-workers ('45) showed that a high dental caries index resulted from feeding to cotton rats a purified diet containing large proportions of simple sugars, and a low index from feeding starch and dextrin. However, Zepplin et al. ('50) found that feeding cotton rats a natural diet resembling the average human diet produced rather severe

dental decay in the presence of only a small amount of added sugar (17%). It has also been observed in this laboratory that although dried whole or skim milk is moderately cariogenic (Anderson et al., '48), fluid whole milk alone or with the addition of 10% simple sugar (Anderson et al., '47), or dried whole milk reconstituted with water (Anderson et al., '48) are almost without cariogenic activity. A preliminary study by Smith ('48) showed that an unrefined syrup when incorporated at low levels (2, 5, 10%) into a synthetic diet in which the remainder of the carbohydrate was floured dextrin produced no less tooth decay than comparable levels of sugar.

In view of the above-cited findings, further studies have been made of the effect of two naturally occurring sugars on the production of dental caries, when incorporated in diets consisting of natural foodstuffs, fluid or dry, or in purified diets.

EXPERIMENTAL

Weanling cotton rats (19 to 21 days of age) from our stock colony were distributed equally by litter mates among the control and experimental groups. All animals received water and food ad libitum and were supplemented each week with two drops of haliver oil. At the end of the experimental period (14 weeks), the animals were killed and their teeth evaluated for lesions according to the method previously described (Shaw et al., '44b).

Four experiments were made. Since Smith ('48) had found that natural syrup increased caries production when fed in dry purified diets, the first experiment was to determine whether this effect remained when the natural syrup was fed in a fluid diet. The basal diet for this experiment consisted of oatmeal and whole milk, as is indicated in table 1. Natural syrup or cerelose was dissolved in the basal diet at a level of 17%. The natural syrup was also fed in the dry state with the basal ration. The fluid diets, which were made up twice daily to maintain their fluid consistency, were homogenized in

TABLE 1

Composition of diets — dry weight basis

INGREDIENTS	EXP. 1				EXP. 2 [1]				EXP. 3			EXP. 4		
	866 (fluid)	867 (fluid)	868 (fluid)	869 (dry)	870 (basal)	871	872	873	875 (basal)	875a	883	874	875 (basal)	876
Oatmeal	50	50	50	50					50	50	50	34	50	66
Milk [2]	33	33	33	33					32	32		48	32	16
Natural syrup no. 1		17		17			23.4		18			18	18	18
Cerelose			17											
Sucrose					23.4					18	18			
Natural syrup no. 2								23.4						
Syrup [3]						23.4								
Casein [4]					24	24	24	24			8.5			
Dextrin					43.6	43.6	43.6	43.6			23.5			
Salts IV [5]					4	4	4	4						
Corn oil					5	5	5	5						

[1] Diets contained 4% 1:20 liver extract at expense of entire ration.

[2] Whole milk was used in diets 866–869; dry weight content given was based on 12.5% solids. In diet 869 and other dry diets containing milk, dried whole milk (Klim) was used.

[3] Made with 20% glucose, 80% sucrose and cooked to approximate viscosity of natural syrups. The syrup was added to the diet on a dry weight sugar basis.

[4] B vitamins added in amounts adequate for growth (Shaw et al., '44b).

[5] Patterned after the salts of milk (Phillips and Hart, J. Biol. Chem., *109:* 657, 1935).

a Waring Blendor, while the dry ration was ground in a Wiley mill until it passed through a medium-fine screen. Iron, copper and manganese were added to each diet at a rate of 1 mg Fe, 0.1 mg Cu and 0.1 mg Mn for each 6.25 gm of milk solids.

The second experiment was to determine the cariogenicity of several syrups and the influence of the physical state of the sugar upon caries production. In this experiment a ration consisting of casein, dextrin, corn oil, salts IV and 23.4% sucrose or other sugars was used (table 1). Two unrefined syrups and a syrup composed of glucose and sucrose cooked to comparable viscosity were used as the experimental ingredients. These ingredients were mixed in the diets at weekly intervals in order to maintain their sticky consistency.

Since the results of the first two experiments suggested that the extensive caries produced by feeding the dry, oatmeal-milk-syrup diet was not due to the greater cariogenicity of the natural syrup, additional experiments were made to study the other components of this diet. In the third experiment, the effect of substituting dextrin for all of the constituents of milk except casein was determined. The basal diet containing the natural syrup was also fed but it was dried and reground in order to eliminate the differences in the physical states of the two rations due to the stickiness of the syrup. The production of caries by the dried syrup diet was compared to the action of a similar diet containing sucrose.

In the 4th experiment the levels of milk and cereal were varied to determine their effect on growth and caries production. The basal diet of this experiment contained 50% oatmeal, 32% dried whole milk, and 18% natural syrup. The cariogenic activity of this basal diet was compared to that of diets containing 16% less or 16% more milk, with the oatmeal content correspondingly increased or decreased. The oatmeal used in these diets was ground in a Wiley mill until it passed through a medium-fine screen.

RESULTS

The data in table 2 show a marked influence of the physical state of the diet on the production of tooth decay. Cotton rats fed a dry, oatmeal-milk-syrup diet had 26 carious fissures per head, while litter mates fed the same diet in fluid form had only one carious fissure per head.

A significant difference between the cariogenicity of natural and refined sugars when studied by controlled animal experiments was not demonstrated. The data in table 3 reveal that although natural syrup No. 1 tends to be slightly less cariogenic, and natural syrup No. 2 and refined syrup tend to be slightly more cariogenic, than sucrose, the caries activities of these sugars are quite similar when they are fed at a level of 23% in a purified diet.

Data on the effect on the production of dental caries of feeding different levels of dried whole milk and cereal are summarized in table 4. Although these animals did not maintain the rate of growth observed in the animals previously fed the basal oatmeal-milk diet (table 1, diet 869), the extensiveness of the decay was similar. One animal which received the basal oatmeal diet (875) died after 7 weeks on experiment. When the teeth of this animal and its litter mate receiving diet 874 and sacrificed at the same time were evaluated, a severe decay equivalent to that of animals fed this oatmeal-milk diet for longer periods was observed. Although severe caries resulted from a diet containing 48% dried whole milk, there was a slight further increase in dental decay when the level of milk was dropped to 32% and the level of cereal similarly increased. Four of the 5 litters experienced this increase in both incidence and extent as the level of milk was decreased from 48% to 32%. Lowering the level of milk further did not result in additional decay. It is of interest to point out that when the level of milk was decreased the amounts of simple sugar, minerals and fat present were also decreased.

The increased caries production with decreased levels of milk was consistent enough to lead to an investigation of the

TABLE 2

Effect of fluidity on the cariogenicity of oatmeal-milk-sugar diets

RATION FED	NUMBER OF ANIMALS	WT. GAIN/WK.			CARIES LESIONS [1]	
		1st mo.	2nd mo.	3rd mo.	Average incidence	Average extent
		gm	*gm*	*gm*		
Basal (866) oatmeal-milk, fluid	6	13	10	6	1	1
Basal + sucrose, fluid	7	12	12	7	1	1
Basal + natural syrup, fluid	7	13	12	7	1	1
Basal + natural syrup, dry	7	14	14	8	25 ± 5	72 ± 21

[1] Incidence equals average number of carious fissures per capita. (The cotton rat has a total of 40 fissures per head, 10 in each quadrant.) Values given in tables are average caries scores and average deviation.

Extent is an expression of the average total caries involvement per capita. Individual lesions are evaluated 1+ to 5+ with increasing severity of lesions; 1+ to 2+ = lesions involving primarily the enamel; 3+ = enamel and a considerable amount of dentine; 4+ to 5+ = lesions resulting in fractures.

TABLE 3

The cariogenicity of natural or refined sugars when incorporated into a purified diet

	NUMBER OF ANIMALS	WT. GAIN/WK.			CARIES LESIONS	
		1st mo.	2nd mo.	3rd mo.	Average incidence	Average extent
		gm	*gm*	*gm*		
Basal (870) — sucrose	11	12	9	5	12 ± 4	28 ± 12
Basal — syn. syrup	11	11	9	7	13 ± 5	31 ± 18
Basal — sucrose	14	12	8	5	11 ± 4	28 ± 14
Basal — natural syrup no. 1	14	14	8	7	9 ± 5	22 ± 14
Basal — sucrose	11	13	9	5	12 ± 3	30 ± 10
Basal — natural syrup no. 2	11	12	10	6	14 ± 5	34 ± 15

TABLE 4

Effect on growth and caries production of feeding various levels of oatmeal and milk in dry natural diets

RATION FED	NUMBER OF ANIMALS	WT. GAIN/WK.			CARIES LESIONS		
		1st mo.	2nd mo.	3rd mo.	Average incidence	Average extent	Average number of fractured fissures
		gm	*gm*	*gm*			
Exp. 1							
Basal (875a) — sucrose	5	9	6	6	26 ± 6	81 ± 27	7
Basal — natural syrup (875)	5	12	8	5	24 ± 5	75 ± 24	8
Basal (875a)	4	8	5	5	24 ± 6	71 ± 24	4
Basal minus milk (883)	4	7	5	5	31 ± 3	109 ± 16	15
Exp. 2							
Basal (875) — 32% milk	5[1]	11	9	5	28 ± 5	83 ± 19	5
Basal + 16% milk	5[1]	12	8	3	25 ± 3	73 ± 12	5
Basal (875)	5	11	8	5	31 ± 3	95 ± 13	5
Basal minus 16% milk	5	10	9	6	27 ± 5	83 ± 21	5

[1] One animal killed after 7 weeks on experiment.

effect on caries production of the various constituents of dried whole milk. The first of this series of studies is reported herein, and the data relative to caries production are contained in table 4. Natural syrup (875) or sucrose (875a), when incorporated in a dry, oatmeal-milk diet, produced similar severe tooth destruction. However, when the dried whole milk was replaced by casein and dextrin (883) a further increase in caries incidence and tooth destruction occurred. Twice as many teeth were fractured, although 73% of the replacement ingredients was floured dextrin and the remainder was fine casein. Both of these nutrients are generally considered to possess low cariogenic activity. Since this latter diet (883) contained considerably less simple sugars, its cariogenicity would normally be expected to be proportionally decreased. It is also noteworthy that this substitution resulted in a much lower fat and mineral content of diet 883 in comparison to the basal diet.

Observations on the gross appearance of the teeth and lesions revealed striking differences between the teeth of animals fed the dry oatmeal-milk diets and those of rats fed the purified diets. There was little or no plaque material or staining on the teeth of the oatmeal-milk animals, despite the more extensive decay. When present, the plaque material was blackish to white. The teeth of cotton rats fed the purified diets were usually heavily coated with a brittle material of deep yellow-brown color. It appeared, therefore, that this material was of dietary origin.

The carious lesions of animals fed purified diets (which in this laboratory routinely contain 4% 1:20 liver extract at the expense of the entire ration) are deeply pigmented with yellow-brown color. As noted in a previous publication (Shaw et al., '44a), this pigmentation facilitates the detection of very early decay. When cotton rats were fed the purified diets, the lesions tended uniformly to begin at the base of the fissure at the narrowest point of the enamel and to spread into the associated cusp. Even in cases of severe decay the lesions tended to be clearly demarcated and the pigmentation

was associated with the softening and opacity caused by the decay. In contrast, the lesions of the cereal-milk animals were only faintly yellow, the depth of color apparently depending in part on the rapidity of attack of the caries process and its duration at the time of sacrificing the animal. Although these lesions tended to originate at the point of the narrower rim of enamel, they frequently occurred at the wide rim, or the softening extended throughout the cusp. It occasionally was possible to push the fine wire, No. 42, dental probe entirely through the thick portion of the cusp. It was impossible to do more than scratch the surface of the teeth of animals fed the purified diets, except in decayed areas. Chalky or opaque areas occasionally were encountered on the thick, labial or lingual enamel surfaces of the cusps. The lesions occurring as a result of feeding the oatmeal-milk diets have been observed to be deeply pigmented; however, this was an infrequent finding.

The jaws and skulls of animals which were fed the low mineral diet 883 were flexible, and readily punctured with a fine wire probe. A few femurs and the skulls and incisors of these animals were available for analysis. Preliminary ashing data revealed the presence of mild rickets. The average per cent ash of the femurs was 65% for animals receiving the purified diet, 63% for those fed 32% milk and 51% for those receiving no milk; while the skulls contained 65%, 60% and 55% and the incisors 76%, 75% and 72% ash, respectively, on an unextracted dried basis. Intact molars were not available for analysis.

The lowest pH obtained when the diets were dissolved in water in increasing amounts was pH 6.6 and 5.2 at saturation for adequate and deficient oatmeal-milk diets, respectively, and 5.8 for the purified diets.

DISCUSSION

The finding of Anderson et al. ('48) that cariogenic substances fed in a fluid diet result in a very low caries score has been confirmed. Our studies on the cariogenicity of natural

foods revealed that a diet consisting of 50% oatmeal, 32% dried whole milk and 18% natural syrup resulted in extensive caries when fed to cotton rats. However, when the same amounts of oatmeal and natural syrup were homogenized in an equivalent level of fluid whole milk and fed to cotton rats for three and one-half months, negligible caries production resulted.

Smith ('48) found that natural syrup, when incorporated into a purified diet at low levels (2, 5, 10%), resulted in a higher caries index than sucrose similarly fed. These data indicate that under some circumstances naturally occurring sugars may be more cariogenic than refined sugars, an observation which is in contrast to many reports in the literature. The cariogenic activity of the natural syrup or of sucrose when fed in dry form in the above oatmeal basal diet was such that little or no difference in the production of tooth decay resulted. This diet has been shown to be highly cariogenic even when it contains a considerably smaller amount of simple sugar. However, the feeding of two kinds of natural sugars (unrefined syrup form) or refined sugars as syrup or in the dry state at a level of 23% in a purified diet with dextrin also showed their cariogenic activity to be similar. These results indicate that naturally occurring sugars in syrup form when fed at a level affording even distribution in the diet are directly proportional in cariogenicity to their sugar content. The results obtained by Smith can probably best be explained on the basis of local accumulation of sugar due to the stickiness of the natural syrup.

The observation made by Zepplin et al. ('50) that natural diets containing 17% sucrose (the amount eaten by the average American) resulted in extensive caries when fed to cotton rats has been confirmed by the studies here reported. A natural diet of oatmeal, milk and natural syrup (18%) produced caries in over 50% of the tooth fissures of the experimental animals. It has further been observed that lowering the level of milk and correspondingly increasing the oatmeal content resulted in increased production of caries, although

by such a procedure the level of simple sugars in the diet was decreased. When the dried whole milk was replaced by crude casein and floured dextrin, a low fat and a low mineral diet resulted which produced even more extensive decay, with a greater incidence of fractured teeth. These changes did not alter the diet consistency or the protein content of the ration. Preliminary ashing data on the bones and incisors showed a definite correlation between ash content and caries. A decreased ash content was correlated with increased caries and with the decreased mineral content of these cereal-milk diets. In view of the implication of minerals in caries production revealed by employing these cereal-milk diets, it is of interest to recall the work of Osborn et al. ('37a, b, c; previously discussed) and the results of Driezen and co-workers ('50), who studied the utilization of cereals by a strain of oral *L. acidophilus*. The latter authors found that in the presence of amylase and of adequate amounts of essential members of the B vitamins, the degree of acid production was for the most part directly related to the degree of refinement of the cereal grain. Belding and Belding ('44) have observed that incubation of oral organisms with refined cereals may cause a transmutation to more pathogenic forms.

On the other hand, Smith ('48), using a purified diet, and Zepplin et al. ('50), employing natural diets, further observed that a 2% level of sucrose was sufficient to initiate dental caries in the cotton rat, and that increasing the level to 10% or 17% resulted in both greater incidence and greater extent. The presence of higher levels of sucrose in the natural diet did not result in a similar proportional increase in caries. Smith also observed that in the presence of 17% sucrose and 67% total carbohydrate in an adequate purified diet, floured dextrin produced twice as much decay as coarse dextrin. These findings indicate that complex carbohydrates under certain conditions may be quite cariogenic. A level of approximately 17% of simple sugar appeared to result in maximum initiation of caries, and to accelerate the breakdown of more complex carbohydrates if they were in a finely divided

form which can be impacted in the narrow fissures of the cotton rat tooth, thus making them more readily available as substrate in the caries process. Our finding of the increased cariogenicity of the oatmeal basal diet when it was altered by substituting floured dextrin and casein for the milk portion suggests further that a lower level of simple sugar will initiate and aggravate caries production in animals fed a low mineral diet.

SUMMARY

Studies on the caries-producing action of natural foods when fed to cotton rats are presented.

A significant difference between the cariogenicity of natural or refined sugars has not been demonstrated. The incorporation of natural sugars into various purified and natural diets produced dental decay similar to that produced by refined sugars fed at equivalent levels.

A highly cariogenic diet consisting of oatmeal, dried whole milk and sugar is described. The importance of the physical state of the diet with respect to the cariogenicity of carbohydrates was shown in that feeding this diet in fluid form resulted in negligible caries scores. Further studies of this diet by altering the levels of various constituents indicated that under certain conditions complex carbohydrates may be as cariogenic as simple sugars.

Substitution of dextrin and casein for the milk of this basal oatmeal-milk diet resulted in increased caries production and in a decreased ash content of the bones, which suggest that minerals may play an important role in the cariogenic potential of diets.

The influence of natural or purified diets on plaque formation and the pigmentation of carious lesions is described. Differences of this type were not correlated with severity of decay but are definitely associated with the type of diet fed.

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EFFECT OF AUREOMYCIN ON THE RESPONSE OF RATS TO VARIOUS FORMS OF VITAMIN B_6 [1]

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Pyridoxal and pyridoxamine are more effective than pyridoxine for the growth of certain microorganisms; and when injected into animals, pyridoxal and pyridoxamine are at least as effective as pyridoxine in promoting growth and in curing acrodynia (Snell and Rannefeld, '45; Sarma et al., '46). However, when incorporated into basal diets, pyridoxal and pyridoxamine appear to be considerably less effective than pyridoxine (Miller and Baumann, '45; Sarma et al., '46). The present study deals with the relative potencies of the three forms of vitamin B_6 in basal diets containing vitamin B_{12} and aureomycin.

EXPERIMENTAL

Two types of deficient diets were used, one containing fibrin and the other DL-methionine. Diet I, which was based on a diet used by Sarma et al. ('46), had the following percentage composition: sucrose 75, blood fibrin 9, vitamin-free casein 9, salts 4, and corn oil 3, plus the following vitamins, in milligrams per kilogram of ration: thiamine 2.0, riboflavin 3.0, nicotinic acid 25, calcium pantothenate 20, 2-methyl-1, 4-naphthoquinone 10, inositol 100, biotin 0.1, folic acid 2.0, and

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choline chloride 1,000.[2] Diet II was similar to diet I except that it contained 18% of vitamin-free casein and, in addition, 0.4% of DL-methionine, making the methionine concentration of this diet approximately equal to that in a diet containing 30% of casein. Several previous studies suggest that methionine increases the need of rats for vitamin B_6 (Sarma et al., '47; Cerecedo et al., '48).

Weanling male rats of the Sprague-Dawley strain 21 days old and weighing 35 to 50 gm, average 45, were placed in single wire bottom cages and given the deficient diet I for a period of two weeks. They were then divided evenly with respect to weight into groups of 5 or 6 animals and placed on the following experimental diets: diet I unsupplemented, diet I plus 20 μg per kilogram of vitamin B_{12}, or diet I plus vitamin B_{12} and 50 mg of aureomycin HCl per kilogram. Each of the three diets was further modified by the addition of 0, 0.25, 0.50 or 0.75 μg of pyridoxine hydrochloride per gram of diet, or of pyridoxal or pyridoxamine equimolar to 0.5 μg of pyridoxine hydrochloride. The rats were fed ad libitum and were weighed weekly during an experimental period of 5 weeks.

When diet II was used, weanling male rats weighing 40 to 50 gm, average 45, were placed immediately on variations of the diet similar to those used for diet I except that the group receiving vitamin B_{12} was omitted. Preliminary studies had indicated that DL-methionine markedly depressed the growth of rats on a diet deficient in vitamin B_6 (see also Sarma et al., '47; Cerecedo et al., '48), and hence no initial depletion was necessary. In the first study an attempt was made to obtain a standard curve by adding 0, 0.5, 1.0 and 1.5 μg of pyridoxine hydrochloride per gram of diet. A linear growth response was found up to the 1.0 μg level of the supplement, with a sharp leveling off at this point. Since these levels of pyridoxine were too high for a satisfactory standard curve, the following amounts were added in subsequent experiments: 0, 0.3, 0.6 and 0.9 μg per gram of diet.

[2] Each rat received two drops per week of a 1:1 mixture of corn oil and halibut liver oil containing 0.5 mg of α-tocopherol per drop.

Pyridoxal and pyridoxamine were assayed at molar equivalents of 0.6 μg of pyridoxine hydrochloride. Since two major series of experiments were performed with each type of basal diet used, the major results to be described were usually observed at least 4 times.

RESULTS

In the absence of vitamin B_6 the rats on the fibrin-containing basal diet I grew from 45 gm at the start of the experiment to an average of 73 gm at the end of the two-week depletion period. Subsequently their rate of growth declined; they gained 8 gm per week during the next three weeks and only 4.5 gm per week during the final two weeks. Symptoms of acrodynia developed during the 6th week in the deficient groups not receiving aureomycin, but no acrodynia developed in the control group that received the antibiotic.

The addition of graded amounts of pyridoxine to the three basal diets produced increasing growth responses. In general, growth in the presence of vitamin B_{12} was almost identical with that in its absence (table 1). The presence of aureomycin in the diet, on the other hand, resulted in marked increases in growth when the level of pyridoxine in the diet was moderately low; 0.25 μg per gram of diet in this series (table 1). At the end of 5 weeks such rats weighed an average of 139 to 141 gm in the absence of aureomycin and 162 gm when the antibiotic was given. This latter weight indicates an apparent pyridoxine content of 0.41 μg per gram of diet; i.e., the presence of aureomycin caused an apparent increase of 64% in the assay value for vitamin B_6. The growth-stimulating effects of the antibiotic were somewhat less both in the absence of any added pyridoxine in the diet and when the levels of pyridoxine in the diet were high. In the presence of aureomycin, pyridoxal and pyridoxamine were as active as pyridoxine in promoting the growth of rats on the fibrin diet (table 1), although in the absence of the antibiotic pyridoxamine was somewhat inferior to pyridoxine, while pyridoxal was markedly inferior.

These effects of aureomycin were particularly evident in rats fed basal diet II containing methionine. With this diet the symptoms of vitamin B_6 deficiency developed more rapidly than on diet I; acrodynia was observed within 4 weeks both in the presence and absence of aureomycin, and the growth rate was low from the beginning of the experiment, so that no preliminary depletion period was necessary. After 5 weeks vitamin B_6-deficient rats on basal diet II weighed 77 gm (table 2), corresponding to an average weekly gain of only

TABLE 1

Effect of vitamin B_{12} and aureomycin on the growth of rats fed diet I and various forms of vitamin B_6[1]

VITAMIN B_6 PER GM DIET	WEIGHT AT 3 WEEKS			WEIGHT AT 5 WEEKS		
	Basal	Basal + B_{12}	Basal + B_{12} and aureomycin	Basal	Basal + B_{12}	Basal + B_{12} and aureomycin
μg	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
None	97	103	103	106	119	124
0.25 pyridoxine·HCl	118	113	127	141	139	162
0.50 pyridoxine·HCl	125	130	138	176	174	190
0.75 pyridoxine·HCl	150	150	162	208	215	219
0.50 pyridoxal·HCl	120	115	132	146	145	188
0.58 pyridoxamine·2HCl	127	125	143	167	171	196

[1] Six rats per group depleted two weeks prior to supplementation. Average weight at end of depletion period, 73 gm.

6 gm. Nevertheless, the addition to this diet of graded amounts of pyridoxine resulted in progressive increments of growth (table 2). Rats receiving 0.9 μg of pyridoxine per gram of diet gained an average of 33 gm per week on this diet (weight at 5 weeks, 211 gm; table 2).

As in the previous study, aureomycin stimulated growth in rats receiving limiting amounts of pyridoxine, although this effect was relatively small when the vitamin was omitted from the diet or when it was present in high amounts. When the diet contained 0.3 μg of pyridoxine per gram, the addition

of aureomycin caused an increase in growth equal to that observed when the pyridoxine content of the diet was doubled (table 2). On basal diet II, as on that containing fibrin, pyridoxal was markedly inferior to pyridoxine for growth, while pyridoxamine was intermediate between the two. However, in the presence of aureomycin growth with the three forms of vitamin B_6 was substantially similar. Thus, the

TABLE 2

Effect of aureomycin on the growth of rats fed diet II and various forms of vitamin B_6 [1]

VITAMIN B_6 PER GM DIET	WEIGHT AT 3 WEEKS		WEIGHT AT 5 WEEKS	
	Basal	Basal + aureomycin + B_{12}	Basal	Basal + aureomycin + B_{12}
μg	*gm*	*gm*	*gm*	*gm*
None	68	78	77	86
0.3 pyridoxine·HCl	102	115	136	158
0.6 pyridoxine·HCl	115	124	159	178
0.9 pyridoxine·HCl	144	143	211	205
0.6 pyridoxal·HCl	102	119	137	169
0.7 pyridoxamine·2HCl	101	123	146	178

[1] Five rats per group, no preliminary depletion period, average initial weight, 46 gm.

percentage increase in growth due to aureomycin was greater when the diet contained pyridoxal than when either of the other two forms of the vitamin was present.

DISCUSSION

The fact that pyridoxal and pyridoxamine incorporated into the diet are inferior to pyridoxine for the growth of rats and chicks has been difficult to understand, inasmuch as the three forms of the vitamin have equal activities when injected, or when administered by mouth but separated from other components of the ration (Snell and Rannefeld, '45; Sarma et al., '46; Miller and Baumann, '45). Rapid absorp-

tion in the upper portions of the intestinal tract when the entire dosage of vitamin B_6 is administered by mouth apart from the ration presumably explains the equivalence of the three forms of vitamin B_6 under these conditions. An explanation suggested for the inequality of response to the three forms of vitamin B_6 incorporated into the diet has been that microorganisms of the digestive tract utilize pyridoxal and pyridoxamine in preference to pyridoxine, and hence more of the latter remains for absorption and use by the rat. A similar explanation has been offered for the diminished utilization of thiamine by human beings fed live yeast (Garber et al., '49).

This explanation can also be applied to the present results. By decreasing the numbers of intestinal microorganisms, or by altering their metabolism in such a way as to decrease the absorption or destruction of vitamin B_6, aureomycin may decrease the proportion of the vitamin ordinarily deflected away from the rat itself, with the resulting increase in growth becoming particularly evident when the amounts of ingested vitamin are suboptimum. If the microorganisms ordinarily utilize pyridoxal or pyridoxamine in preference to pyridoxine, as postulated, then the prevention of this use by an antibiotic should elicit particularly great increases in growth, such as have now been observed when suboptimum amounts of these compounds were fed. In support of this idea are the findings that many lactic acid bacteria, which normally comprise the largest single group of bacteria in the intestinal flora (Porter and Rettger, '40; Shapiro and Sarles, '49; Shapiro et al, '49), require vitamin B_6 for growth, utilize pyridoxal and pyridoxamine in preference to pyridoxine, and may absorb amounts of these vitamins far in excess of their requirements for growth (Snell and Rannefeld, '45; Holden et al., '49).

Contrary to much prevailing opinion, the small intestine of both rats (Porter and Rettger, '40) and chicks (Shapiro and Sarles, '49; Shapiro et al., '49) contains appreciable numbers of viable bacteria, predominantly of the lactic group. According to the concept presented here, these organisms com-

pete successfully with the mucosa for the absorption of vitamin B_6, or, conversely, a substantial proportion of the ingested free vitamin (when administered with the diet) escapes absorption until it reaches the lower parts of the digestive tract.

In most published studies on growth responses due to antibiotics, the diets fed have been relatively crude and complete, and emphasis has been placed on the effect of the antibiotic on utilization of newer growth factors such as vitamin B_{12}. The present results show that an antibiotic can stimulate growth when the amounts of a familiar vitamin are limiting. The suggested mechanism by which aureomycin increases the effectiveness of vitamin B_6 may well apply also to other known vitamins and to yet unidentified accessory food factors.

The present results raise several questions as to the meaning of a bioassay for vitamin B_6. If one wishes to determine the actual amounts of the three forms of the vitamin, the best animal assay would appear to be that in which the basal diet contains a suitable antibiotic. But since the diets of men and farm animals do not ordinarily contain aureomycin, the physiological benefit to be expected from the vitamin B_6 in a particular foodstuff would appear to be better expressed by the results of a conventional assay in which no antibiotic is used, even though, or precisely because, the three forms of vitamin B_6 yield unequal responses in this assay. Similarly, the presence of an antibiotic in a substance being assayed for vitamin B_6 could cause erroneous results as far as the "amount present" is concerned, but they would not necessarily be erroneous in terms of the physiological benefit to be expected.

The use of methionine in the assay diet for vitamin B_6 offers several advantages. With this addition it is not necessary to use fibrin in the basal diet, since ordinary amounts of vitamin-free casein suffice. The need for the vitamin, as shown by lack of growth and symptoms of acrodynia, is so great that no preliminary depletion period is necessary, yet the growth responses to graded doses of the vitamin are

approximately linear. However, before a general recommendation of this diet can be made, it should be determined to what extent the depressive effect of methionine can be overcome by substances other than vitamin B_6.

SUMMARY

1. In agreement with earlier results, rats fed pyridoxal or pyridoxamine incorporated into the basal ration grew less than rats fed equimolar amounts of pyridoxine. The addition of vitamin B_{12} to the basal ration did not alter the relative activities of the three forms of vitamin B_6.

2. Aureomycin increased the growth of rats fed low levels of pyridoxine. This increase was sufficient to cause over-all errors of 10 to 100% in a bioassay for vitamin B_6, but largely disappeared when high levels of the vitamin were fed.

3. Aureomycin caused a marked increase in the growth of rats fed limiting amounts of pyridoxal or pyridoxamine. In the presence of the antibiotic the three forms of vitamin B_6 were approximately equal in growth-promoting activity.

4. Rats fed an assay diet containing 18% of casein and 0.4% of methionine showed linear growth responses to graded doses of vitamin B_6. No preliminary depletion period was necessary.

5. It is postulated that aureomycin may act to prevent utilization or destruction of the growth-promoting nutrient, vitamin B_6, by intestinal microorganisms, thus increasing the amount available to the rat, and that a similar action directed toward other known and possibly unknown food factors may explain in part the growth-promoting effects of antibiotics in crude rations.

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